

MBL

Volume 191

Number 1

THE BIOLOGICAL BULLETIN



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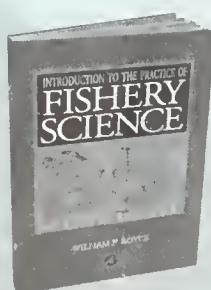
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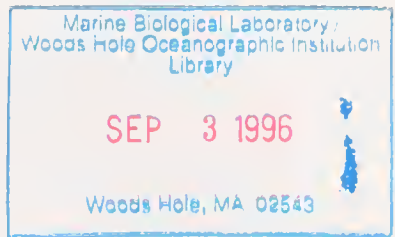
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AUGUST, 1996

Printed and Issued by
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THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to Subscription Manager, THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543. Single numbers, \$38.00. Subscription per volume (three issues), \$95.00 (\$190.00 per year for six issues).

Communications relative to manuscripts should be sent to Michael J. Greenberg, Editor-in-Chief, or Pamela L. Clapp, Managing Editor, at the Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543. Telephone: (508) 289-7428. FAX: 508-457-1924. E-mail: pclapp@mbi.edu.

THE BIOLOGICAL BULLETIN is indexed in bibliographic services including *Index Medicus* and MEDLINE, *Chemical Abstracts*, *Current Contents*, and *CABS (Current Awareness in Biological Sciences)*.

Printed on acid free paper.
effective with Volume 180, Issue 1, 1991.

POSTMASTER: Send address changes to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543.

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ISSN 0006-3185

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Contractile Connective Tissue in Crinoids

RÜDIGER BIRENHEIDE AND TATSUO MOTOKAWA

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Active movements in animals are usually attributed to cellular protein engines, e.g., the actin-myosin system of muscle cells. Here we report the first evidence of an extracellular contractile connective tissue, which we have found in sea lilies and feather stars (Echinodermata, Crinoida). These marine animals have arm muscles that are antagonized, not by other muscles, but by ligaments consisting of extracellular fibrils interspersed with neuron-like cell processes. Contractile cells are lacking, yet these arm ligaments actively contracted upon stimulation. The ligaments stayed in a contracted condition even after the stimulus had stopped. The stresses generated were lower than those of typical skeletal muscles. Additional data from crinoid cirri, which lack muscles entirely, corroborate the hypothesis that the connective tissue of the ligaments is contractile.

Crinoids use their arms for filter feeding, crawling, and swimming. The arms consist of a series of ossicles interconnected by mobile joints (Figs. 1a, 2a). Muscles bend the arms orally (upwards). The aboral (downward) powerstroke has been thought to be effected by the passive elastic recoil of aboral ligaments (1); but we provide evidence here that the ligaments contract actively and thus contribute to the movements of the arms.

At rest, crinoids are anchored by a different set of appendages—the cirri (Fig. 1). Each cirrus consists of a row of ossicles joined by ligaments that lack any muscles (2, 3); yet these appendages move actively (3). We show in this study that the cirri can develop forces that are probably responsible for their movements.

Specimens of crinoids were trawled from a depth of 130 m off Numazu, Suruga Bay, Japan (sea lily *Metacrinus rotundus*) or collected in shallow waters near Kominato, Chiba Prefecture, Japan (feather star *Comanthus*

japonicus). The animals were kept in circulating seawater aquaria. Pieces of arms consisting of 5 to 10 ossicles were prepared as follows: The oral half of each ossicle was removed along with its connecting ligaments and muscles; thus the remaining aboral half ossicles were connected only by their aboral ligaments (Fig. 2b). The mechanical responses—i.e., displacement (Fig. 3a)—and generated forces (Fig. 3b) of these half-arm preparations were examined.

Transmission electron microscopy revealed no evidence of any muscle cells in the ligaments of the arms or cirri; these ligaments appeared to be conglomerates of collagen fibrils, microfibrils, and neuron-like cell processes (Fig. 1b). The neuron-like cell processes were distributed randomly among the fibrils; the cell bodies associated with these processes are located in the pore space of the ossicles and are connected to the nervous system (1). They are likely to be the site of the mechanism that triggered the contraction of the ligaments described below. These findings reconfirm earlier reports that the ligaments of crinoid arms (1, 4, 5, 6) and cirri (2, 3) lack muscle cells.

When arm pieces were connected to a holder at one end and a metal plate was fixed to their other end (Fig. 2b), they showed a constant slow bending under the influence of gravity. But Figure 3a shows the strong flexion against the force of gravity caused by K^+ depolarization; the ligaments shortened to less than half of their length. Contraction of the aboral ligament is the only possible means by which this flexion could have been accomplished. K^+ depolarization had no effect on samples anesthetized with seawater containing menthol, but reactivity was restored after washing in seawater. Probably the K^+ ions acted by depolarizing neural elements (7).

The possibility that the neuron-like cell processes contract upon stimulation has to be considered. Many echi-

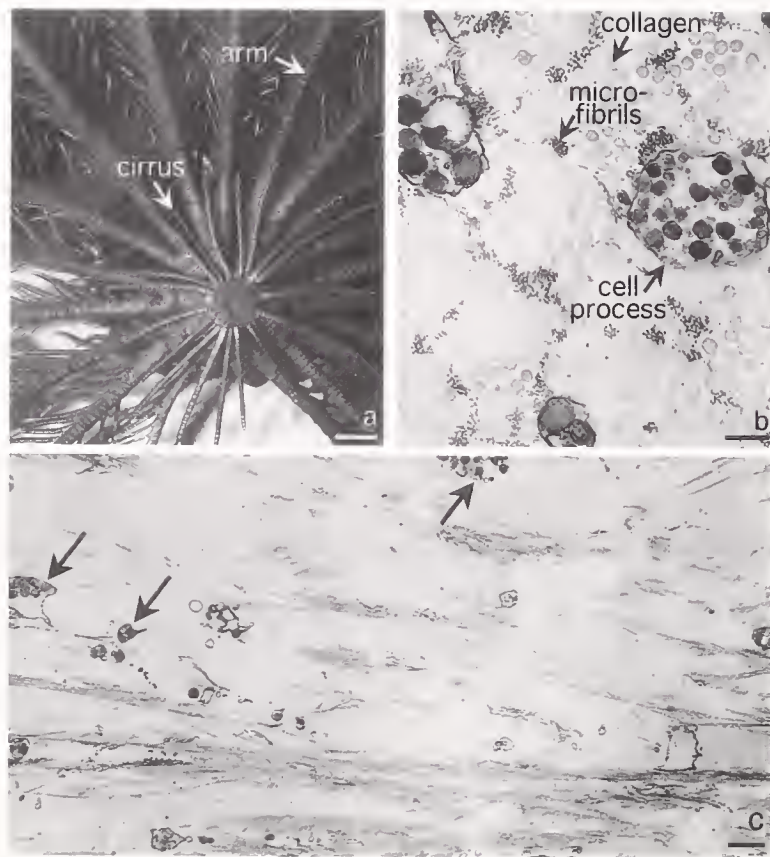


Figure 1. (a) Photograph of a feather star from the aboral side (underside). The long arms radiate from the central body and consist of a series of small ossicles connected by ligaments and muscles. Feather stars can walk or swim with their arms. The smaller cirri serve to anchor the animal when it is resting. Scale bar 5 mm. (b) Transmission electron micrograph of an aboral arm ligament of a feather star fixed after flexion of the arm and processed according to standard methods. This cross section shows that the ligament consists only of collagen, microfibrils, and neuron-like cell processes filled with dark vesicles. Scale bar 350 nm. (c) Low-power transmission electron micrograph of a longitudinal section of the aboral arm ligament of a sea lily. The ligament consists mainly of extracellular fibril bundles that run straight between two ossicles of the arm. Profiles of cell processes are distributed randomly and never run straight. Often the processes contain dark vesicles (arrows). Scale bar 1 μ m.

noderm connective tissues have a structure similar to that of the crinoid ligaments and contain neuron-like cell processes (8, 9). But these processes do not seem to be specialized for contraction, and contractility has never been observed in such tissues. Moreover, the cell processes follow a tortuous path through the ligament and are connected neither to the fibrils nor the ossicles. Therefore we believe that the contractile force causing flexion of our tissue preparations can only be ascribed to the extracellular matrix with its microfibrils and collagen fibrils.

Figure 3b shows the force generated by an arm piece after K^+ depolarization. Often the force fluctuated for some time, suggesting that the force-generating mechanism is an active process, not just a passive recoil of some

elastic element. In force measurements, any possibly elastic element in the ligament is subject to the same tension. If the ligament relaxes to a certain passive force (tension), every mechanical element in the ligament has relaxed to that force. In such a case, an elastic element cannot exert a force higher than that to which it has relaxed. Therefore, if a relaxation is followed by an increase of force, as shown in Figure 3b, it must be ascribed to the active generation of force. In our experiments, the ligaments eventually developed a maximal force that decreased to a lesser, constant value. The maximal force of a single ligament was calculated to be about 3 mN, corresponding to a stress of about 5.6 KPa. Skeletal muscles of vertebrates typically develop stresses of 200 KPa, two orders of magnitude higher.

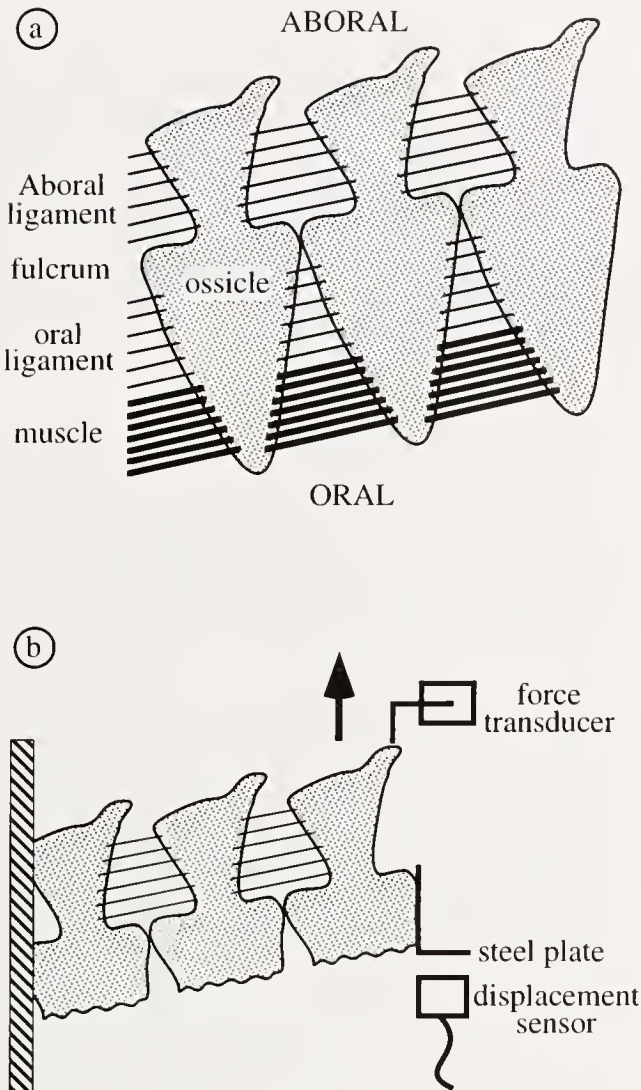


Figure 2. (a) Schematic longitudinal section of a segment of an arm of a feather star. Ossicles are movable against each other at a fulcrum, and they are connected with aboral ligaments, oral ligaments, and muscles. (b) Pieces of arm were glued to a holder oriented with the aboral side up (as in Fig. 2a). The oral part, including muscles and oral ligaments, was removed so that the ossicles were only connected *via* the aboral ligaments. Active flexion occurred in the direction of the arrow and was detected with an eddy current sensor monitoring movements of a stainless steel plate (weight about 110 mg in seawater) fixed to the free end of the preparation. In another set of experiments, forces were measured with a force-gauge whose pick-up needle was positioned on the sample. All experiments were done in a trough containing the experimental solutions at 20°C.

Contractile connective tissue stayed in the contracted state for hours even when the K^+ depolarizing solution was washed out after only 200 seconds. Probably this effect is due to the so-called "connective tissue catch," a phenomenon typical of echinoderm connective tissues

(8). Such tissues can change their stiffness under nervous control and thus serve to lock extremities in given positions over a long time (10). In crinoids, such connective tissues were found in the cirri (3, 7) and in the stalk (11). The arm ligaments go one step further: they not only change passive stiffness, but also develop active contractile forces under nervous control. In summary, arms can be locked in any position by the catch mechanism of the ligaments. Movement can occur when the ligaments soften: then the arms can be moved orally by muscles, and aborally by a ligament that combines elastic properties with the ability to contract actively.

Observations on the cirri of sea lilies corroborate the hypothesis of contractile connective tissue. We have connected the proximal end of freshly dissected cirri to a

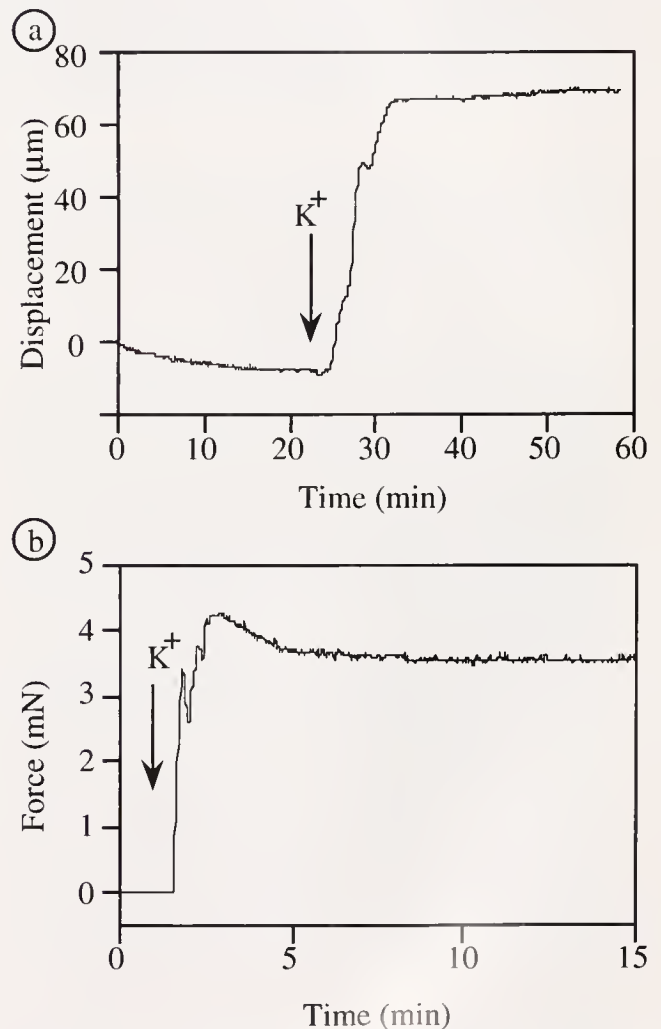


Figure 3. (a) Artificial seawater with elevated potassium concentration (100 mM) caused an upward flexion of a piece of arm of a feather star. (b) Force generated by a piece of arm of a feather star on stimulation with excess K^+ .

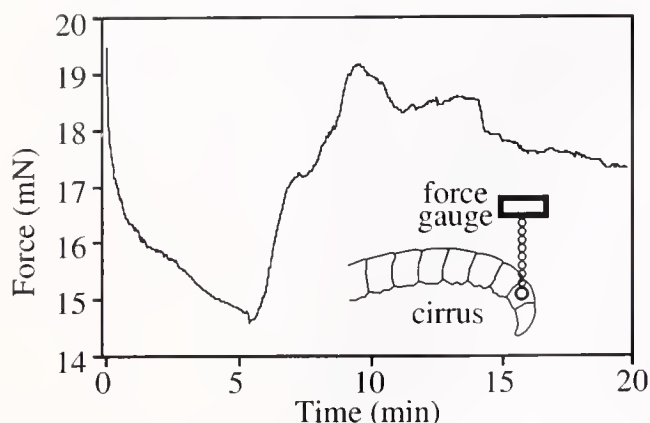


Figure 4. Freshly dissected cirri were fixed at the proximal end, and the distal end was connected *via* a silver chain to a force transducer. A microdrive connected to the force transducer caused the cirrus to bend aborally (upward). For the curve shown the cirrus was bent until the force reached about 20 mN. This position was fixed, and the cirrus was left in aerated seawater without further stimulation. A typical initial phase of stress relaxation was followed by a spontaneous production of force that fluctuated irregularly.

static holder and the distal end, *via* a clip and a chain, to a force transducer in stress-relaxation tests. We observed fluctuating forces spontaneously generated by the cirri (Fig. 4). As noted above, cirral ligaments lack any potential contractile cells, so the observed forces are probably developed by the extracellular matrix. Here, as in the arm ligaments, fluctuating forces strongly suggest an active generation of force and not a passive recoil. Thus, crinoids seem to use contractile ligaments in various parts of their body. Probably a contractile ligament is less energy-consuming than a muscle, so—despite its slow response and the comparatively low forces that it produces—it might be of adaptive significance for a filter-feeding animal.

Active contractility has been proposed for the holothurian dermis (12). But the holothurian dermis contains coelomic pouches lined by muscular tissue that might be responsible for contractions. Force-generating connective tissue in the wall of vertebrate blood vessels (which also contains muscle cells) has recently been proposed on the basis of circumstantial evidence (13). The present study provides the first unequivocal evidence of a contractile connective tissue. We believe that our findings have great importance for both animal biology and medicine.

Acknowledgments

We thank Dr. S. Kikuchi for providing feather stars and Mr. R. Ohki and Mr. T. Chiba for assistance with electron microscopy. The valuable comments of two anonymous referees helped to improve our manuscript significantly. This work was supported by a Sasakawa Research Grant of the Japan Science Society to R.B. and a grant of the Ministry of Education, Science, Sports, and Culture of Japan to T.M.

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Analysis of the Calcium Transient at NEB During the First Cell Cycle in Dividing Sea Urchin Eggs

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Abstract. Accumulating evidence from several systems suggests that nuclear envelope breakdown (NEB) is triggered by an endogenous transient of free calcium. Using *h*- and *f*-semisynthetic aequorins as cytosolic calcium indicators, we have clearly and regularly visualized a single large, global calcium transient just before first NEB in normally developing, monospermic *Lytechinus* eggs. Although similar transients were not observed at NEB in subsequent cell cycles, microinjection of the calcium buffer BAPTA into one blastomere of the two-celled embryo resulted in the inhibition of NEB.

The NEB transient in the first cell cycle was some five-fold smaller than the one associated with egg activation. Our data suggest that this transient takes the form of a calcium wave that spreads inwards from the periphery of the egg toward the nucleus. We confirmed that these NEB transients did not require extracellular Ca^{2+} . In polyspermic eggs, NEB-associated transients were four-fold larger than in monospermic eggs and were periodically repeated. Examination of the distribution of fluorescein-conjugated aequorins with a laser scanning confocal microscope indicated that aequorin both enters the nucleus and is evenly distributed within the cytosol of the egg. The use of *h*- and *f*-aequorins did not reveal any NEB transients during subsequent cell cycles, nor did we detect transients associated with other cell cycle events. However, a complex train of calcium transients in the form of both localized pulses and propagated waves

was detected from embryos beginning at about the morula-to-blastula transition and continuing through to hatching.

Introduction

The molecular basis of cell division appears to be remarkably similar in organisms as diverse as yeasts, sea urchins, and mammals (Murray and Hunt, 1993). However, the roles of key proteins such as cyclin B, p34, and cdc 25 are better understood than those of critical small molecules and ions such as Ca^{2+} . The regulation of a large number of cellular processes by Ca^{2+} suggests that it may also interact in cell cycle control. Indeed, evidence is accumulating that Ca^{2+} may regulate such events as pronuclear migration, pronuclear fusion, nuclear envelope breakdown (NEB), mitotic spindle formation, the transition between metaphase and anaphase, chromosome separation, and cytokinesis (Ciapa *et al.*, 1994; Gillot and Whitaker, 1994; Fluck *et al.*, 1991; Whitaker and Patel, 1990; Steinhardt and Alderton, 1988; Poenie and Steinhardt, 1987; Poenie *et al.*, 1985). But clear experimental evidence in support of the contention that Ca^{2+} transients directly regulate many of these events is still lacking (Hepler, 1994; 1992; 1989).

The argument for an association between a Ca^{2+} transient and a cell cycle event is strongest in the case of NEB (Tombs *et al.*, 1992; Browne *et al.*, 1992; Whitaker and Patel, 1990; Poenie *et al.*, 1985). NEB and chromatin condensation can be induced in sea urchin eggs by microinjecting free Ca^{2+} or IP_3 (Twigg *et al.*, 1988; Patel *et*

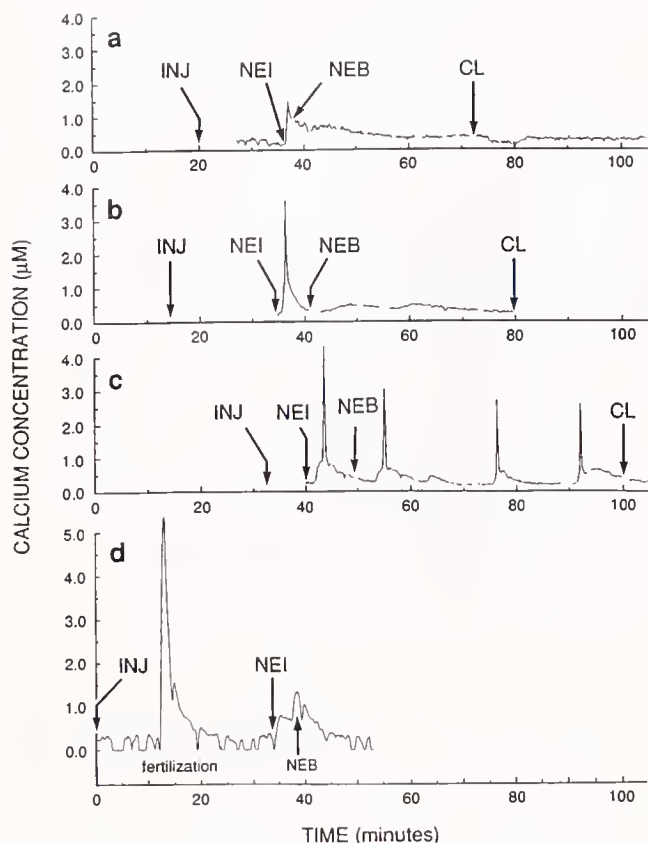


Figure 1. Representative examples of calcium transients preceding NEB in *Lytechinus variegatus* eggs. Zero time indicates fertilization. INJ indicates the time of aequorin injection (in d, aequorin was injected before fertilization). Breaks in the record indicate DIC observations. Because the fertilized eggs were observed intermittently with DIC optics to detect nuclear envelope breakdown and cleavage, the times indicated for these events reflect when they were observed, rather than the precise time at which they occurred. NEI indicates the time closest to the transient at which the nuclear envelope was seen to be still intact; NEB, nuclear envelope breakdown, and CL, cleavage. (a) A monospermic egg cultured in ASW. (b) A monospermic egg cultured in Ca^{2+} -free ASW. (c) A polyspermic egg cultured in ASW. Both polyspermic eggs and the monospermic controls in this experiment displayed first cleavage at around 100 min, rather than the 70 to 80 min often observed. (d) Comparison between the calcium transient at activation and that at NEB.

al., 1989a, Steinhardt and Alderton, 1988) or by photo-activating $[\text{Ca}^{2+}]_i$ transients in nitr-5 loaded cells (Kao *et al.*, 1990). Furthermore, if the calcium buffers EGTA and BAPTA (Steinhardt and Alderton, 1988; Twigg *et al.*, 1988) or a precipitating antibody or a specific inhibitor to the multifunctional Ca^{2+} /calmodulin-dependent protein kinase (Baitinger *et al.*, 1990) are microinjected into fertilized and parthenogenetically activated sea urchin eggs, NEB and further progress through mitosis are blocked. In addition, monospecific antibodies to intra-

cellular calcium pumps (Silver, 1986; Hafner and Petzelt, 1987) and antagonists (ryanodine and 8-(diaminoethyl)octyl-3,4,5-trimethoxybenzoate) to intracellular Ca^{2+} channels (Silver, 1989) have also been shown to block NEB and the subsequent mitotic cycle.

Using fluorescent Ca^{2+} indicators such as Fura-2 or Ca^{2+} -Green, several studies have presented evidence of fluctuations of $[\text{Ca}^{2+}]_i$ in the cell cycle of ammonia-activated and fertilized sea urchin eggs, including a transient that corresponded to the time of NEB (Ciapa *et al.*, 1994; Whitaker and Patel, 1990; Patel *et al.*, 1989b; Poenie *et al.*, 1985). Similar signals have been reported in starfish (Stricker, 1995), cultured mammalian cells (Poenie *et al.*, 1986; Tombes and Borisy, 1989; Kao *et al.*, 1990), and fertilized mouse eggs (Tombes *et al.*, 1992). Although attempts to correlate Ca^{2+} transients with NEB have been unsuccessful in the eggs of *Xenopus* and zebrafish, failure to detect a transient in these systems might be attributed to the techniques used, rather than to the absence of a calcium signal (Rink *et al.*, 1980; Busa and Nuccitelli, 1985; Reinhard *et al.*, 1995).

Thus, while the weight of the evidence does indeed suggest that a Ca^{2+} transient precedes and regulates NEB, many questions remain. What initiates the transient? What form does the transient take (*i.e.*, is it a propagating wave)? Is the transient unique to the first cell cycle? What are the molecular targets of the transient?

We decided, therefore, to further investigate the NEB calcium transient in *L. variegatus* using new nontoxic, semisynthetic recombinant forms of the chemiluminescent calcium indicator aequorin, a complementary methodology to fluorescent calcium reporters (Miller *et al.*, 1994; Shimomura *et al.*, 1993, 1990). Using the highly sensitive *h*- and *f*-aequorins, we have consistently visualized a single global calcium transient just prior to NEB in the first cell cycle in monospermic *Lytechinus* eggs. In polyspermic eggs, significantly larger, repetitive Ca^{2+} transients were observed. The NEB calcium transient is not dependent on external Ca^{2+} , and preliminary data suggest that it takes the form of a wave that spreads from the periphery of the egg towards the nucleus. These data are interpreted in light of recent results relating to independent Ca^{2+} regulation in the nucleus (Himpens *et al.*, 1994). A short preliminary report of some of the data has appeared previously (Browne *et al.*, 1992).

Materials and Methods

Preparation of gametes

Eggs and sperm of *Lytechinus variegatus* (supplied by the Duke Marine Laboratory) were obtained either by intracoelomic injection of 0.5 M KCl or by electrical stimulation (12-V DC). Eggs that had been washed sev-

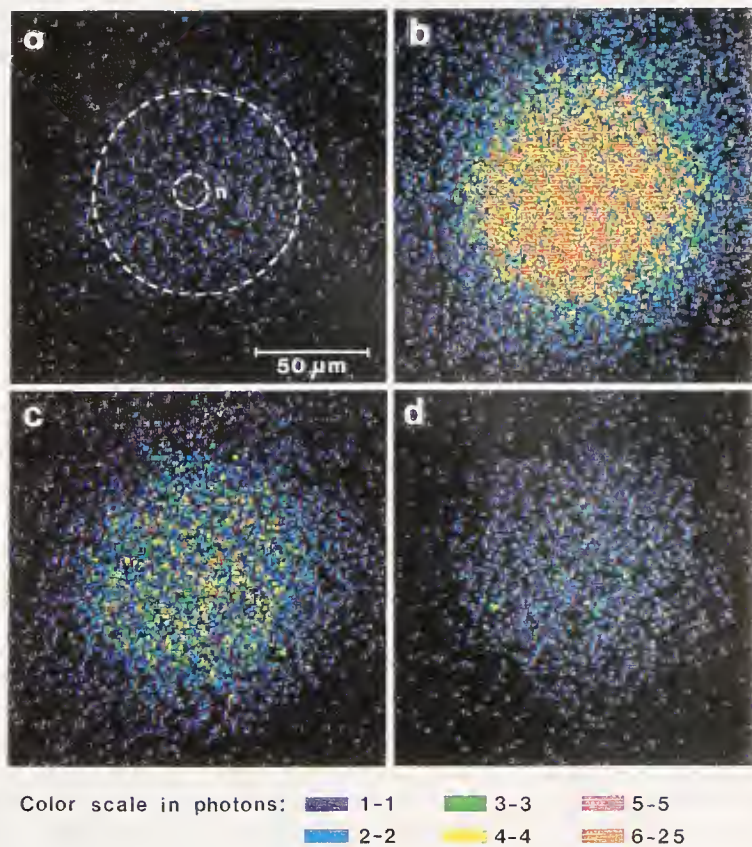


Figure 2. Visualization of the NEB calcium transient shown in Figure 1a. The panels show successive 3-min periods of accumulated luminescent light beginning 35 min after fertilization. The resting level of $[Ca^{2+}]$, within this egg is shown in panel a. A pulse of elevated calcium precedes NEB, fills the whole egg, and lasts for about 5 min (panels b–c). By panel d, $[Ca^{2+}]$, has nearly returned to the resting level. The dashed boundaries indicate the locations of the nucleus (n) and the cell membrane.

eral times in artificial seawater (ASW—Marine Biological Laboratory, Woods Hole, MA) were fertilized by the addition of 10 μ l of concentrated sperm in ASW to 100 ml of egg suspension. After fertilization, the eggs were again washed in either ASW or calcium-free ASW containing 1 mM EGTA. Jelly coats were removed by vacuum filtration of the eggs through a 100- μ m nylon mesh.

Microinjection into eggs

Fertilized eggs were microinjected with either *h*-aequorin, *f*-aequorin, or a fluorescein-conjugated aequorin, obtained from Dr. O. Shimomura of the Marine Biological Laboratory. The *h*- and *f*-aequorins are reported to have 16 and 20 times, respectively, the relative intensity (at pCa 7) of recombinant aequorin *in vitro* (Shimomura *et al.*, 1993). These aequorins were injected as a 1% solution in 50 mM KCl, 0.1 mM EDTA, 1 mM MOPS buffer, at pH 7.1. Fertilized eggs were microinjected in

chambers consisting of a coverslip fragment mounted with beeswax to form a wedge on a 22-mm-square #0 coverslip. Microinjection was carried out by the method of Hiramoto (1962) and Kiehart (1982) but was modified for aequorin as outlined in detail by Miller *et al.* (1994). The injection volume was about 40 pl, less than 10% of the egg volume.

To compare the NEB calcium transient to that at activation, unfertilized eggs were loaded into gelatin-coated wedge chambers and microinjected with aequorin as described above. ASW containing active sperm was introduced into the outer part of the Hiramoto chamber. The time needed for sperm to swim to the eggs in the inner part of the chamber was more than sufficient to allow us to switch our imaging system to a photon-counting mode and thus monitor the initiation of the activation wave.

Luminescent imaging

The calcium-dependent luminescent light from *h*- and *f*-aequorin-loaded eggs was projected onto the photo-

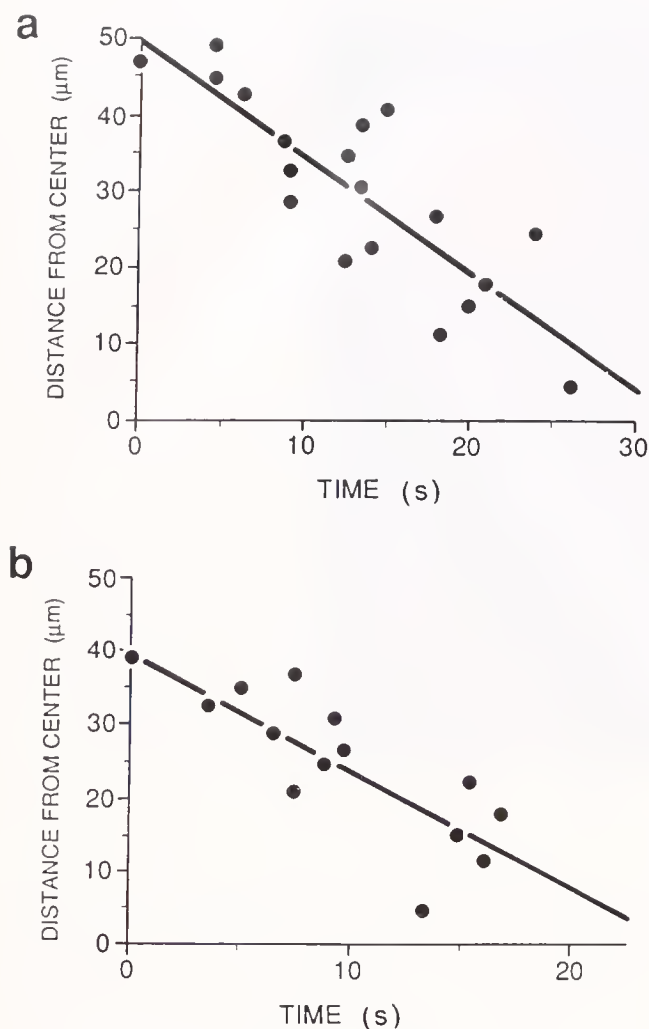


Figure 3. Graphs in a and b illustrate data from two experiments that yielded the most light at NEB. Each egg profile was divided into concentric areas representing equal volumes of the egg. The times of maximal photon count rate (in photons/coordinate/second) were determined for each concentric area and plotted against the distance of the midpoint of each area from the center of the egg. A linear regression fit of each plot yielded the estimated speed of a wave peak starting at the periphery of the egg and converging on the center (or nucleus). The two plots yield wave velocities of 1.54 and 1.49 $\mu\text{m/s}$.

cathode of an imaging photon detector (IPD) by a Zeiss Axiovert 100 TV microscope platform with an image port on the same axis as a Zeiss 63 $\times$ 1.25-NA Plan-Neofluor objective. The IPD system collects photons and directly digitizes the x - y coordinates and time of each photon in a sequential data file. Such files were later used to reconstruct images over any desired time interval (described in detail in Miller *et al.*, 1994). Photon collection was intermittently interrupted so we could observe the eggs directly with DIC (differential interference contrast) optics, and the DIC images were stored on videotape.

The temporal and spatial correlation between the aequorin luminescence and morphological events was determined from analysis of computer files and videotaped records. After most experiments, the egg chamber was perfused with 1% Triton X-100 in ASW to lyse the eggs. The resultant luminescence enabled us to estimate the residual amount of photoactive aequorin. Embryos that were not lysed were continually imaged until hatching, thus highlighting the nontoxic nature of these new ultrasensitive aequorins.

Fluorescent imaging

Fluorescein-conjugated aequorin was microinjected into fertilized eggs as described above. The eggs were examined by confocal fluorescence microscopy with a Zeiss LSM 410 Axiovert at the Zeiss Microscopy Facility at the Marine Biological Laboratory. Images were obtained with a 40 $\times$ 0.6-NA Achroplan lens and Zeiss LSM software. Other than an initial adjustment of brightness and contrast, these images were not processed further. Photographic records were stored on 35-mm film.

Data analysis of calcium transient

With the aequorin method, one is essentially limited by the total amount of signal, rather than the signal-to-noise ratio. When the NEB signal was integrated over periods of a few minutes, this was not a problem. But to facilitate a more sophisticated analysis to determine the kinetics of the calcium rise and explore the possibilities of wave propagation, we had to generate an analysis routine suited to our limited signal. Moreover, our imaging equipment (Miller *et al.*, 1994) is essentially two-dimensional and has no resolution in the z -axis. This is a serious limitation when observing a signal generated within a spherical volume. We devised an analysis routine in which each egg profile was divided into concentric areas representing equal volumes of the egg. Each concentric area was identified by the distance, in micrometers, between its midpoint and the center of the egg (or the nucleus, if the latter did not happen to be located in the center of the egg).

To determine whether the NEB signal took the form of a wave, the times of maximal photon count rate (in photons/coordinate/second) were determined for each concentric area and plotted against the distance of the midpoint of each area from the center of the egg. Such a plot should determine (1) whether a wave does indeed propagate, (2) the direction of propagation, and (3) the velocity of propagation. A linear regression fit of each plot yielded the estimated speed of a wave peak starting at the periphery of the egg and converging on the center (or nucleus).

Table 1

Comparison of the calcium transients at activation (Fert) and nuclear envelope breakdown

Egg #	Counts at Fert*	Counts at NEB*	Counts at lysis*	[Ca ²⁺] at Fert**	[Ca ²⁺] at NEB**	$\frac{[Ca^{2+}]_{NEB}}{[Ca^{2+}]_{Fert}}$ ** (%)
1	63.8	0.53	104.4	4.94	0.78	15.8
2	279.4	6.32	345.0	5.40	1.26	23.3
3	120.1	4.20	250.7	4.13	1.14	27.6
4	243.0	2.93	529.7	4.83	0.88	18.3
Avg	176.6	3.50	307.5	5.07	1.06	21.3

*Milliphotons/coordinate/second.

**Assuming luminescence rises to the 2.6 power of [Ca²⁺] and the resting levels before and after fertilization are 0.10 μ M and 0.25 μ M respectively.

Calibration of aequorin luminescence signal

Estimations of free [Ca²⁺]_i levels during both activation and NEB transients were made on two assumptions: (1) the resting level of [Ca²⁺]_i before activation is 0.10 μ M, whereas after activation, but prior to NEB, it increases to 0.25 μ M (Whitaker and Patel, 1990; Baitinger *et al.*, 1990; Poenie *et al.*, 1985; Steinhardt and Alderton, 1988); and (2) the luminescence of *h*- and *f*-aequorin varies with Ca²⁺ concentration *in vivo* as it does *in vitro*. From plots #2 and #3 (*i.e.*, those for *h*- and *f*-aequorin) in figure 1 in Shimomura *et al.* (1993), we estimate that between pCa 7 and pCa 6.5 luminescence rises to the 2.6 power of [Ca²⁺].

Injection of BAPTA buffer during the second cell cycle

We used the microinjection technique described above to inject BAPTA (1,2-bis[*o*-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid) buffer into one of the two daughter blastomeres after the first cell division, but before the second NEB. From our previous experience with BAPTA-type shuttle buffers (Fluck *et al.*, 1994; Miller *et al.*, 1993; Speksnijder *et al.*, 1989) and from data reporting the effects of BAPTA on NEB in the first cell cycle in *L. pictus* (Steinhardt and Alderton, 1988; Twigg *et al.*, 1988), we estimated that a final cytosolic concentration of about 10 mM BAPTA should have a significant effect, if a calcium transient is involved in triggering second NEB. The injection solution contained 70 mM BAPTA; 80 mM KCl; 48 mM CaCl₂; 3.4 mM MgCl₂, and 8 mM HEPES, set to a pH of 7.0. Injection volumes were between 20 and 50 pl (*i.e.*, less than 10% of the total blastomere volume).

Results

NEB calcium transient

Monospermic eggs in ASW. In 42 cases of *f*- or *h*-aequorin-loaded, monospermic *L. variegatus* eggs that de-

veloped in ASW, all but one showed a striking calcium transient that began soon before first NEB (Figs. 1a and 2). Throughout the experiments, photon collection was interrupted periodically to collect and store DIC images of eggs onto a video record. Examination of the video record showed that in every case the calcium transient immediately preceded or corresponded to NEB. In three experiments in which video images were acquired every 30 s, NEB was seen to start about a minute after the initiation of the calcium transient. The calcium rise occurred in about 10 s and reached $1.9 \pm 0.3 \mu$ M (s.e.m.). After the NEB transient, the calcium levels usually fell back to near the pre-NEB resting level in about 5 min. As shown in Figure 2, the transient was global; *i.e.*, it eventually filled the whole egg.

In monospermic eggs injected with *h*- or *f*-aequorin, no transients were detected at NEB in subsequent cell cycles nor at any other phases of the first or subsequent cell cycles. These negative results were not due to exhaustion of the aequorin. Lysis of the eggs in Triton X-100 after each experiment resulted in photon emission, which indicated that the eggs still retained abundant active aequorin, with count rates often exceeding thousands of photons per second for several minutes.

Monospermic eggs in calcium-free ASW. In four cases, aequorin-injected eggs were cultured in calcium-free ASW containing 1 mM EGTA. These gave similar single, global transients at NEB (Fig. 1b) that peaked at $3.2 \pm 0.4 \mu$ M (s.e.m.), a signal some twofold larger than was observed in monospermic aequorin-injected eggs at NEB.

Polyspermic eggs in ASW. In four cases, aequorin-loaded eggs cultured in natural seawater proved to be polyspermic, as judged by observations of the number of asters and of subsequent cell division patterns. These appeared in four batches of eggs on four different days and most likely resulted from the fusion of two or more sperm with otherwise normal eggs. The NEB pulse

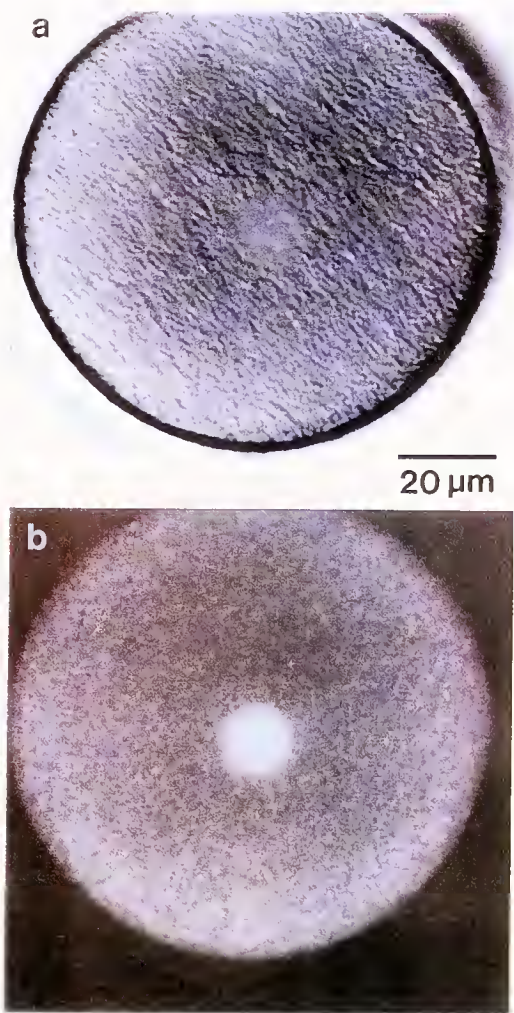


Figure 4. Aequorin distribution in the egg of *Lytechinus variegatus* microinjected with fluorescein-conjugated aequorin at about 20 min after fertilization. (a) Brightfield light micrograph of the egg indicating the position of the nucleus. (b) Corresponding confocal fluorescent image of a 10- μ m optical section in the equatorial region of the egg. Other than an initial adjustment for brightness and contrast, the images were not processed.

heights in these polyspermic eggs averaged $7.6 \pm 1.0 \mu\text{M}$ (s.e.m.), a value around four times larger than the average in the monospermic eggs. Moreover, these polyspermic transients were always clearly repeated, decreasing in intensity over time. In the most remarkable case, the transient was repeated 14 times; in two others, 5–6 times; in the fourth, the three additional transients were smaller compared to the first one but still unmistakable (Fig. 1c). These repeated transients were not correlated with any observable structural change within the egg. Despite these large and repeated transients, cleavage began at about the same time in all of these eggs, as it did in aequorin-loaded, monospermic ones from the same batch.

This confirms an earlier report in which polyspermy did not significantly delay cleavage (Timourian and Watchmaker, 1971). However, several of the subsequent cleavages in these polyspermic eggs eventually regressed, in part perhaps because of a combination of their polyspermy and their slight flattening in the Hiramoto/Kiehart chamber.

Nature of the NEB calcium transient

Because the total amount of calcium-dependent light generated at first NEB was limited, the pattern of calcium release was examined only in those eggs that yielded the largest signals at NEB. Only 7 of the 47 eggs that were injected with aequorin had a photon emission per unit area per second that was large enough to permit an effective analysis of the pattern of photon emission. Figures 3a and b are examples of two of these. Five of the seven eggs analyzed gave similar results; however, the remaining two were not statistically significant. The two plots shown yield wave velocities of 1.54 and 1.49 $\mu\text{m/s}$, with fits of 0.65 and 0.71, respectively, both statistically significant at the $P < 0.01$ level.

Comparison of NEB and calcium transients associated with egg activation

In four experiments in which calcium transients at NEB were compared to those at fertilization, the $[\text{Ca}^{2+}]_i$ peak at activation was about five times larger than at NEB (Table I). Eggs were imaged through first cleavage and lysed by the addition of 1% Triton X-100 in ASW. In all cases, the large luminescent signal resulting from the influx of extracellular $[\text{Ca}^{2+}]$ (not shown) indicated that the smaller NEB signal was not due to a lack of active aequorin. Figure 1d shows a representative plot of $[\text{Ca}^{2+}]_i$ against time of an egg injected with aequorin prior to fertilization.

Distribution of aequorin in cytosol and nucleus

To determine the cytoplasmic and nuclear distribution of aequorin prior to NEB, fluorescein-conjugated aequorin was microinjected into fertilized eggs (Fig. 4a,b). Equatorial brightfield and fluorescent optical sections taken through a representative *L. variegatus* egg showed an intense fluorescent signal from the nucleus. This clearly indicates that aequorin ($M_r \geq 20,000$ to 22,000) microinjected into the cytosol enters the nucleus. The increased intensity of the signal from the nucleus compared to that from the cytosol probably reflects the larger amount of aequorin in the nucleus due to an absence of inclusions. The cytosolic distribution of the aequorin appeared to be homogeneous, except for

Table II

Inhibition of second nuclear envelope breakdown (NEB) by the microinjection of BAPTA into one blastomere at the two-cell stage

Egg #	Time of 1st cleavage	Injection time	NEB in control blastomere	NEB in injected blastomere	Cleavage in control blastomere	Cleavage in injected blastomere
1	66	75	yes	no	116	no
2	65	71	yes	no	116	no
3	68	80	yes	no	115	no
4	66	84	yes	no	115	no
5	67	90	yes	no	115	no
6	67	74	yes	no	113	no
7	68	81	yes	no	113	no
8	68	84	yes	no	113	no

The second blastomere serves as a control. The final cytosolic concentration of buffer was 10 mM. Times are given in minutes after fertilization.

slightly more intense fluorescence in the cortex than in the endoplasm. There was no indication that cytosolic aequorin was compartmentalized.

Effects of BAPTA injection on second NEB

The failure to visualize a Ca^{2+} transient in subsequent cell cycles raises a question as to its role in later NEBs. Calcium signals may actually be present, but of significantly lower intensity and not detectable by this method. Silver (1989) injected the calcium buffer BAPTA into one of two blastomeres of the two-celled sand dollar embryo, inhibiting NEB in the injected blastomere. In a similar procedure, injection of BAPTA buffer to a final cytosolic concentration of 10 mM into one cell of the two-celled sea urchin embryo blocked second NEB and subsequent cleavage in the injected blastomere (Table II). This suggests that, in spite of our failure to visualize a calcium signal with the aequorins and equipment at our disposal, a transient elevation of $[\text{Ca}^{2+}]_i$ is required for the second NEB.

Post-morula to blastula calcium transients

Aequorin luminescence was monitored in six monospermic eggs developing in natural seawater for more than 17 h. Although no detectable light was seen above the resting level during the subsequent cell divisions, striking patterns of luminescence reappeared after about 5 h. Periodically thereafter, a train of strong signals was recorded until the embryos hatched and swam out of view of the detector. These signals were often localized to specific regions of the embryo and took the form of either waves or nonpropagating transients (Fig. 5). Because these data consist of luminescent signals but no video records, we were unable to precisely correlate these signals with specific developmental events. However, the presence and intensity of these transients confirms that

the failure to detect any further calcium signals during cell cycles following the first mitotic cycle was not due to aequorin exhaustion.

Discussion

We consistently observed a global Ca^{2+} transient preceding NEB during the first cell cycle in monospermic *L. variegatus* eggs. This observation supports the gathering evidence that an endogenous calcium transient is needed to trigger at least the first NEB. In monospermic eggs, we found peak values of $[\text{Ca}^{2+}]_i$ at first NEB of around $1.9 \mu\text{M}$. Assuming a pre-NEB cytosolic resting level of $0.25 \mu\text{M}$, this represents about an eightfold rise in $[\text{Ca}^{2+}]_i$. When comparing NEB $[\text{Ca}^{2+}]_i$ peaks to those at egg activation, we found them to be around fivefold smaller. Our data are also consistent with reports that the source of this Ca^{2+} appears to be intracellular stores, since no extracellular Ca^{2+} is necessary for this signal (Poenie *et al.*, 1985).

In Table III we have summarized our data, as well as published data, reporting calcium transients at activation and first NEB in a variety of sea urchin species. In general, smaller transients have been reported when fluorescent calcium probes have been used. We suggest two explanations for this observation: (1) If present in sufficient quantity, these high-affinity calcium reporters may buffer $[\text{Ca}^{2+}]_i$ levels in the same range as the resting level of $[\text{Ca}^{2+}]_i$. (2) They also tend to saturate below micromolar concentrations of Ca^{2+} *in vivo* (Diliberto *et al.*, 1994). A combination of these effects would dampen both the activation and NEB calcium transients. The use of aequorins avoids these problems (Miller *et al.*, 1994). Differences in experimental protocols, detection equipment, calibration procedures, and species examined may also contribute to the range of values displayed.

Our observation of larger NEB transients in polysper-

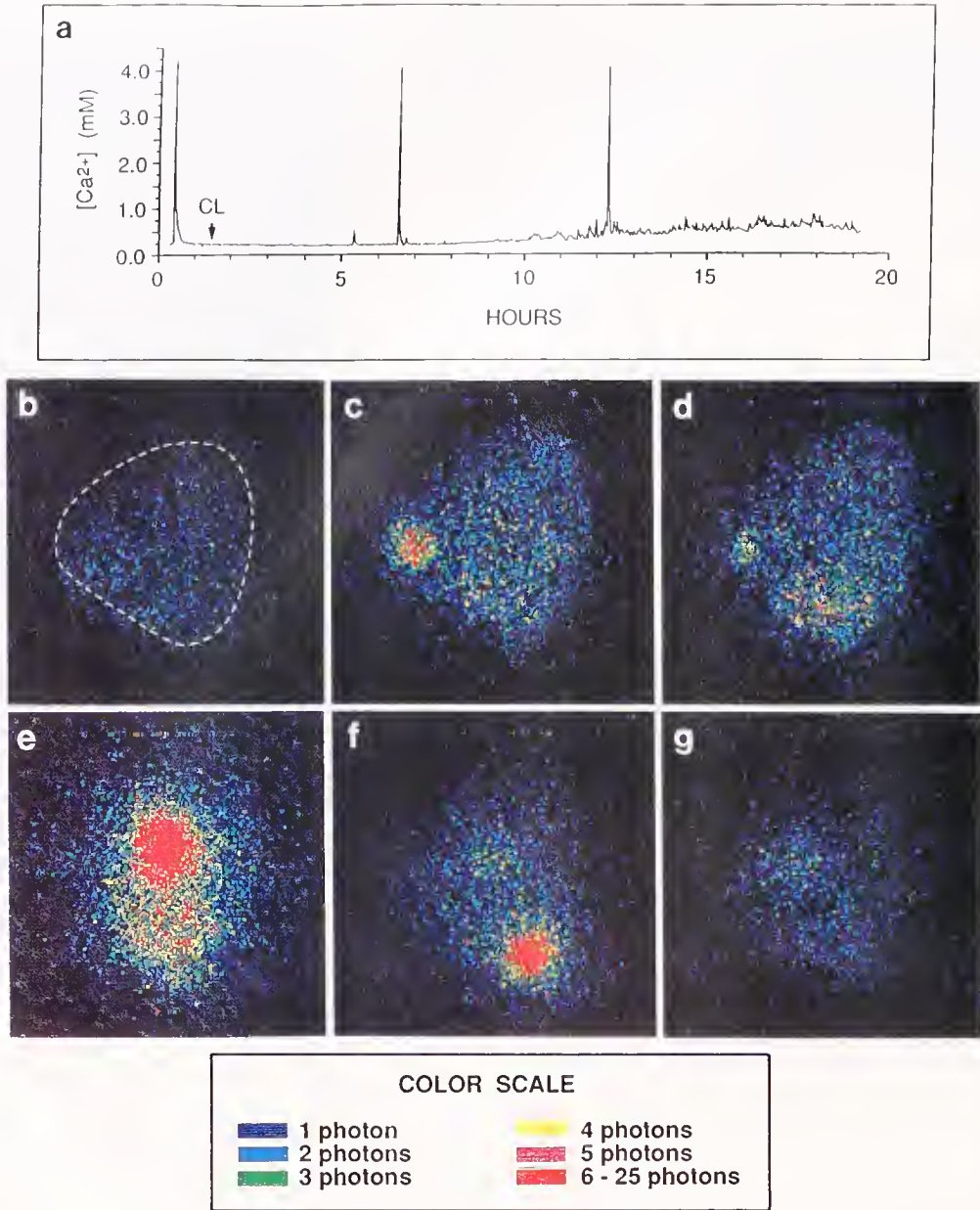


Figure 5. Calcium transients from a *Lytechinus variegatus* egg during the first 17 h of normal development. The first pulse corresponds to NEB in the first cell cycle. CL indicates first cleavage. The absence of any NEB transients, or indeed any cell-cycle-related transients, in subsequent cell cycles is apparent for several hours, even though the egg is developing normally. After 5 h, at about the morula-to-blastula transition, a few transients occur. This is followed, beginning about 10 h later, by a steady rise in [Ca²⁺], over the whole embryo. This general rise appears to be due to a train of calcium transients that continue up to the time that the ciliated embryo swam out of the field of view of the detector. (b) Visualization of the data shown above. Each panel represents 20 min of accumulated light beginning 10 h after fertilization. Because a corresponding DIC record is lacking, these patterns of calcium release are difficult to correlate with developmentally significant events (see text). Clearly, however, the absence of signal following the first cell cycle is not due to the exhaustion of aequorin.

mic eggs is also consistent with what has been reported (Whitaker and Patel, 1990). Although this is the first report to link repetitive transients with polyspermy in sea

urchin eggs, Stricker (1995) has reported numerous repetitive "secondary fertilization signals" in polyspermic starfish eggs. The observation of repetitive Ca²⁺ pulses in

Table III

Comparison of our results with published data reporting calcium transients at activation and nuclear envelope breakdown (NEB) in a variety of sea urchin species in the genus *Lytechinus* and one species of *Arbacia*

Species	Reporter	Activation (Ca ²⁺ μ M)	First NEB (Ca ²⁺ μ M)	Reference
<i>L. variegatus</i>	<i>h</i> -aequorin, <i>f</i> -aequorin	5.07	1.9	This paper
<i>L. pictus</i>	Fura-2, dextran	ND	0.4 ¹	Ciapa <i>et al.</i> , 1994
<i>L. pictus</i>	Ca ²⁺ -green, dextran	2.0 ²	ND	Gillot and Whitaker, 1994
<i>L. pictus</i>	Fura-2	1.5 ³	ND	Galione <i>et al.</i> , 1993
<i>L. pictus</i>	Fura-2	1.0–5.0	0.35	Whitaker and Patel, 1990
<i>L. pictus</i>	Fura-2	ND	0.566	Steinhardt and Alderton, 1988
<i>L. pictus</i>	Fura-2	2.5	ND	Hafner <i>et al.</i> , 1988
<i>L. pictus</i>	natural aequorin	1.3–8.0	ND	Swann and Whitaker, 1986
<i>L. pictus</i>	Fura-2	1.95	0.404	Poenie <i>et al.</i> , 1985
<i>L. pictus</i> , <i>A. punctulata</i>	natural aequorin	1.0	ND	Eisen <i>et al.</i> , 1984
<i>L. pictus</i>	natural aequorin	2.5–4.5	ND	Steinhardt <i>et al.</i> , 1977

ND = No data.

¹ Authors report a mean transient increase of 0.85 μ M from what appears to be a resting level of between 0.2 and 0.3 μ M.

² From figure 1d.

³ From figure 4c.

polyspermic eggs might provide a clue to the mechanism that initiates and propagates transients in monospermic eggs. It has been suggested that periodic calcium transients are generally triggered by repetitive overloading of the lumen of the endoplasmic reticulum with calcium (Jaffe, 1993), and that centrosomes organize calcium-rich endoplasmic reticulum (Wolniak *et al.*, 1980; Henson *et al.*, 1990; Terasaki and Jaffe, 1991). Injected centrosomes have also been reported to induce parthenogenesis of *Xenopus* oocytes (Schiebel and Bornens, 1995). Recent evidence also suggests that, in the embryonic cell, a sperm-derived factor plays a role in the generation of cell-cycle-related calcium transients, including the one at NEB (Carroll *et al.*, 1994; Kono *et al.*, 1995; Osawa *et al.*, 1994). Together, these point to the possibility that some additional factors introduced by extra sperm somehow contribute to triggering these repeated calcium transients.

The NEB transient during the first cell cycle quickly fills the whole egg. Our working hypothesis was that some sort of calcium-wave propagation was involved. In none of the 42 examples we examined, however, did we see an NEB transient that crossed an egg from pole to pole as a wave. This suggested that if the transient was propagating as a wave, it had to be traveling from the outside towards the nucleus, or *vice versa*. Our analysis was designed to explore these possibilities.

Although our analysis was circumscribed by limited signal and a lack of resolution in the z-axis, the data indicate that the NEB transient takes the form of a calcium wave that begins in the outer regions of the egg and

spreads inward toward the central nuclear region with a velocity of about 1.5 μ m/s. We have not as yet explored possible propagation mechanisms of this wave. A suggestion might be calcium-induced calcium release (CICR). The wave velocity determined by our analysis is slow compared to some reports of waves propagated by CICR (Jaffe, 1993). It represents, however, an average velocity from the periphery to the center. A situation might exist in which the wave moves at different velocities in different regions of the egg. This phenomenon has been reported during activation in sea urchin eggs, when the wave moves faster in the cortex than it does in the central region of the egg (Mohri and Hamaguchi, 1991; Shen and Buck, 1993).

The best immediate hope for further exploring the nature of this wave would appear to lie in fluorescent confocal microscopy. Using this technique, Gillot and Whitaker (1994) recently reported two additional Ca²⁺ transients that follow the activation transient in *L. pictus*: the first coincided with the beginning of pronuclear migration; the second with pronuclear fusion. This report did not, however, continue to include a description of the transient at NEB. Stricker (1995), using ratio imaging and confocal microscopy, reported postfertilization waves in starfish embryos, but he was unable to demonstrate that these signals took the form of a wave.

The location of initiation and the direction of the wave suggest that the first cell cycle may be triggered by a cortical biological clock, such as the one described by Hara *et al.* (1980). The latter had the same periodicity as the cell division cycle in *Xenopus* embryos and functioned

in the absence of any nuclear material. We suggest that, at least for the first cell cycle, there may be an analogous situation in the sea urchin egg.

We failed to visualize any NEB calcium transients in later cell cycles, but we could consistently block second NEB with the calcium buffer BAPTA. A plausible explanation might be that after the first cell cycle the NEB calcium transients are restricted to the nuclear or perinuclear region rather than the whole cytosol. The nucleus clearly possesses all the component parts (endomembranes, sequestered calcium, receptors, channels, pumps, etc.) to transduce a localized calcium signal (Minamikawa *et al.*, 1995; Himpens *et al.*, 1994). This idea of a progression from global to localized transients is supported by a study from the sand dollar *Echinarracnius parma*, in which a calcium transient was reported to precede second NEB by around 6 min, to last about 40 s, and to be restricted to the perinuclear region (Silver, 1994; Silver *et al.*, 1994). Our failure to detect localized transients in subsequent cell cycles of *L. variegatus* might be explainable as follows: if these signals were as intense as the first global transient we recorded, but were restricted to a volume 10 μm in diameter, they would result in only about 0.1% of the luminescent emission seen from the whole egg during first NEB.

The first cell cycle may be unique, and signal transduction pathways might be different in subsequent cycles. An understanding of calcium signaling in subsequent cycles is limited by the fact that most data have been collected from, and are relevant to, only the first cell cycle. In mouse embryos the ability to induce global Ca^{2+} transients is a unique property associated with the early NEBs and is lost in subsequent cell cycles (Kono *et al.*, 1995). Sea urchin embryos might exhibit a similar condition in which NEB is preceded by a global calcium transient in the first cell cycle but is lost in subsequent cell cycles. However, in somatic cells such as Swiss 3T3 fibroblasts, global rather than local Ca^{2+} transients are seen to precede NEB (Kao *et al.*, 1990).

The trigger for Ca^{2+} release, the targets involved, and thus the overall mechanism by which Ca^{2+} might regulate NEB are currently under investigation. An IP_3 receptor has been shown to be present in the nuclear envelopes of several cell types (Bachs *et al.*, 1992; Matter *et al.*, 1993), providing a mechanism for the initial localization of Ca^{2+} fluxes at the nuclear envelope. Sea urchin eggs have been shown to possess IP_3 -sensitive and -insensitive calcium stores (Clapper and Lee, 1985; Turner *et al.*, 1986; Oberdorf *et al.*, 1986; Swann and Whitaker, 1986), as well as a calcium-induced calcium release mechanism mediated by a ryanodine receptor (Buck *et al.*, 1992; Fujiwara *et al.*, 1990; Galione *et al.*, 1991, 1993; Gerasimenko *et al.*, 1995; Lee *et al.*, 1993; Mc-

Pherson *et al.*, 1992; Rakow and Shen, 1990). In addition, IP_3 and the calcium-binding proteins calsequestrin, calreticulin, and calsequestrin-like protein have been localized throughout the cytoplasm of sea urchin eggs (Parys *et al.*, 1994; Lebeche and Kaminer, 1992; Henson *et al.*, 1990). In *Xenopus*, overexpression of calreticulin inhibits the occurrence and frequency of repetitive Ca^{2+} wave activity (Camacho and Lechleiter, 1995). The calcium transient, be it global or localized, could function through the multifunctional Ca^{2+} /calmodulin-dependent protein kinase that has been identified in sea urchin eggs and shown to be necessary for NEB (Baitinger *et al.*, 1990).

In prophase-arrested oocytes of both mouse and starfish, NEB during germinal vesicle breakdown does not need to be preceded by a calcium transient (Tombes *et al.*, 1992; Witchel and Steinhardt, 1990). Thus, it is premature to draw any broad conclusions as to the absolute requirement for a pre-NEB calcium trigger or the distinction between global or localized transients. Tombes *et al.* (1992) suggest that because germinal vesicle breakdown appears to be Ca^{2+} -independent, whereas NEB is Ca^{2+} -dependent, there may be regulation by more than one signaling pathway. This would not be surprising, as it is becoming obvious that many important signaling pathways associated with key events in early development have redundant mechanisms (Galione *et al.*, 1993).

Acknowledgments

Supported by NSF grant BIR-9211855 to ALM, and NIH GM43264 to REP. We thank Drs. O. Shimomura, S. Inouye, and Y. Kishi for providing *lt*-aequorin, *f*-aequorin, and fluorescein-conjugated aequorin.

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Evolution of Intragonadal Development in the Diminutive Asterinid Sea Stars *Patiriella vivipara* and *P. parvivipara* with an Overview of Development in the Asterinidae

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Abstract. The diminutive asterinid sea stars *Patiriella vivipara* and *P. parvivipara* incubate their embryos in the gonads to the advanced juvenile stage. Despite the small size of their eggs (135–150 μm diameter), development is lecithotrophic. Development proceeds through the wrinkled blastula, gastrula, and brachiolaria larval stages. The gastrulae and larvae are uniformly ciliated and swim, propelled by the cilia, in the gonadal fluid. The brachiolaria is pear-shaped and has a vestigial brachiolar attachment complex composed of three small brachia. At no stage in development are the embryos attached to the gonad. Metamorphosis occurs as the larvae swim in the gonadal lumen. Internal development involves formation of one large enterocoel at the anterior end of the archenteron and one small posterior enterocoel on the left side of the archenteron. The archenteron closes to form the rudiment for the adult gut. As a result of the small size of the egg and the nonfeeding mode of development, the larvae of *P. vivipara* and *P. parvivipara* are minute, about 270 μm and 210 μm in length, respectively. Newly metamorphosed juveniles are about 240 μm and 310 μm in diameter, respectively. Post-metamorphic development involves substantial growth of the juveniles, which leave the parent at a diameter between 1.0 and 5.0 mm. The presence of a vestigial brachiolar complex and lecithotrophic development indicates that these species had a free-living lecithotrophic brachiolaria in their ancestry. We suggest that the evolution of viviparity in *Patiriella* sp. involved retention of a large egg by an ancestor that had a lecithotrophic brachiolaria followed by a secondary reduction in the size of the

ovum and simplification of the larva. The range of life histories seen in *Patiriella* is atypical of asteroid genera and supports the contention that the evolution of viviparity and other modes of modified development in the Asteroidea follows phylogenetic lineages.

Introduction

Asteroid echinoderms have diverse larval forms that are broadly divided into planktotrophic feeding larvae and nonfeeding lecithotrophic larvae. Possession of a small egg and a planktotrophic larva is generally accepted to be the ancestral mode of development in the Asteroidea, with evolution of nonfeeding development associated with acquisition of a large egg (Strathmann, 1978, 1993). An exception to this dichotomy is the viviparous development of the asterinid sea stars *Patiriella vivipara* and *P. parvivipara* (Byrne, 1991, 1996; Chia and Walker, 1991; O'Loughlin, 1991). These species have small eggs (135–150 μm diameter) similar in size to the eggs of a *Patiriella* species (*P. regularis*) that has planktotrophic development and considerably smaller than the eggs of *Patiriella* species that have lecithotrophic development (Table 1; Byrne and Barker, 1991). With the intragonadal location of the embryos, however, development of *P. vivipara* and *P. parvivipara* is clearly not planktotrophic. Despite the small size of their eggs, *P. vivipara* and *P. parvivipara* give birth to large (1.0–5.0 mm diameter) juveniles that are up to 30% of the diameter of the parent (Byrne, 1991, 1996). This substantial post-metamorphic growth is supported by intragonadal cannibalism.

Viviparity in *P. vivipara* and *P. parvivipara* is associated with the suite of life history traits often associated

Table 1

Size of eggs, larvae and newly metamorphosed juveniles of *Patiriella* species (SE, n)

Species	Oocyte: diameter (μm)	Brachiolaria larva: length (μm)	Newly metamorphosed juvenile: diameter (μm)
<i>Patiriella regularis</i>	150 (0.8, 20)	1430 (3.9, 20)	408.4 (9.0, 20)
<i>Patiriella calcar</i>	415 (3.7, 60)	746.9 (17.5, 10)	516.8 (18.3, 10)
<i>Patiriella gunni</i>	400 (1.9, 60)	490 (23.4, 12)	NA
<i>Patiriella exigua</i>	390 (4.3, 60)	690 (14.6, 7)	580 (18.9, 20)
<i>Patiriella vivipara</i>	148 (0.7, 21)	270 (8.1, 17)	314 (21.5, 12)
<i>Patiriella parvivipara</i>	135* (0.2)	207 (11.9, 12)	244 (9.3, 37)

NA, data not available.

* Largest primary oocytes seen in gonad.

with brooding in echinoderms (Byrne, 1991, 1996). Both of these species are small and hermaphroditic. *P. vivipara* has a maximum arm radius (R) of 15.0 mm, and *P. parvivipara*, the smallest known sea star, has a maximum radius of 5.0 mm (Dartnall, 1969; Keough and Dartnall, 1978; Byrne, 1996). Fertilization occurs in the gonads, most of which are ovotestes. The simultaneous presence of mature eggs and sperm in the gonads of *P. vivipara* and *P. parvivipara* creates the potential for self-fertilization.

Associated with their low-dispersal life history, *Patiriella vivipara* and *P. parvivipara* have the most restricted distribution known for the Asteroidea. *P. vivipara* is endemic to southeast Tasmania where it is recorded from four locations (Dartnall, 1969). *P. parvivipara* is restricted to the west side of the Eyre Peninsula in South Australia where it is recorded from five locations (Keough and Dartnall, 1978). Both species

have a geographic distribution of 150–300 km. These species are intertidal and inhabit about 100 m of the shoreline at each location where they occur. *P. vivipara* and *P. parvivipara* are morphologically similar to the sympatric species *P. exigua* and, prior to the observation of parturition, were considered to be variants of that species (Dartnall 1969, 1971; Keough and Dartnall, 1978). Systematists consider *P. vivipara* and *P. parvivipara* to be sibling species (Dartnall, 1971; Keough and Dartnall, 1978).

P. vivipara and *P. parvivipara* form part of a sympatric series of *Patiriella* species distributed around the Australian coast from Western Australia to north Queensland (Dartnall, 1971). The range of developmental patterns exhibited by *Patiriella* spans the broadcast-brooding continuum seen in the Asteroidea and is documented in a series of studies (Lawson-Kerr and Anderson, 1978; Byrne, 1991, 1992, 1995, 1996; Byrne and Barker, 1991; Chen and Chen, 1992; Byrne and Anderson, 1994; Cerra and Byrne, 1995a, b). Comparative life history data are available for eight species. *P. regularis* has the ancestral pattern of development through typical planktotrophic bipinnaria and brachiolaria larvae (Byrne and Barker, 1991). *P. gunni*, *P. calcar*, *P. brevispina*, and *P. pseudoexigua* develop through lecithotrophic planktonic brachiolariae, and *P. exigua* develops through a modified lecithotrophic benthic brachiolaria (Lawson-Kerr and Anderson, 1978; Byrne, 1991, 1992, 1995; Chen and Chen, 1992; Byrne and Anderson, 1994; Cerra and Byrne, 1995a, b). Documentation of the intragonadal development of *P. vivipara* and *P. parvivipara* in this study provides comparative data at the most derived end of this life-history series. With these contrasting modes of development, *Patiriella* presents an ideal model with which to

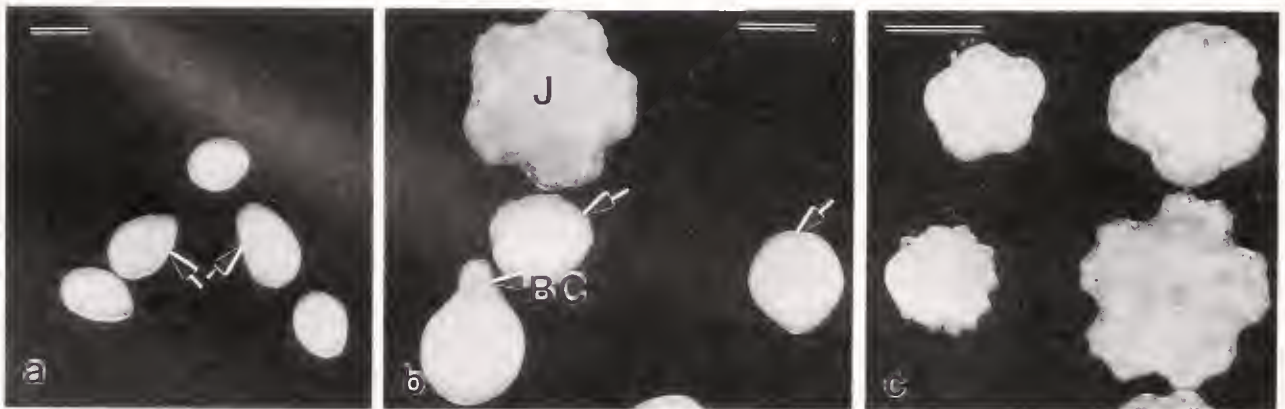


Figure 1. Light microscopy of development of live specimens of *Patiriella vivipara*. Scale bars = 160 μm . (a) Pear-shaped late gastrulae/early brachiolaria (arrows) freed from the gonad. (b) Metamorphosing larva (bottom left) with the brachiolar complex (BC) at the anterior end. Metamorphosis in the other larvae (arrows) is nearly complete. J, juvenile. (c) Metamorphosing (left) and recently metamorphosed juveniles (right).

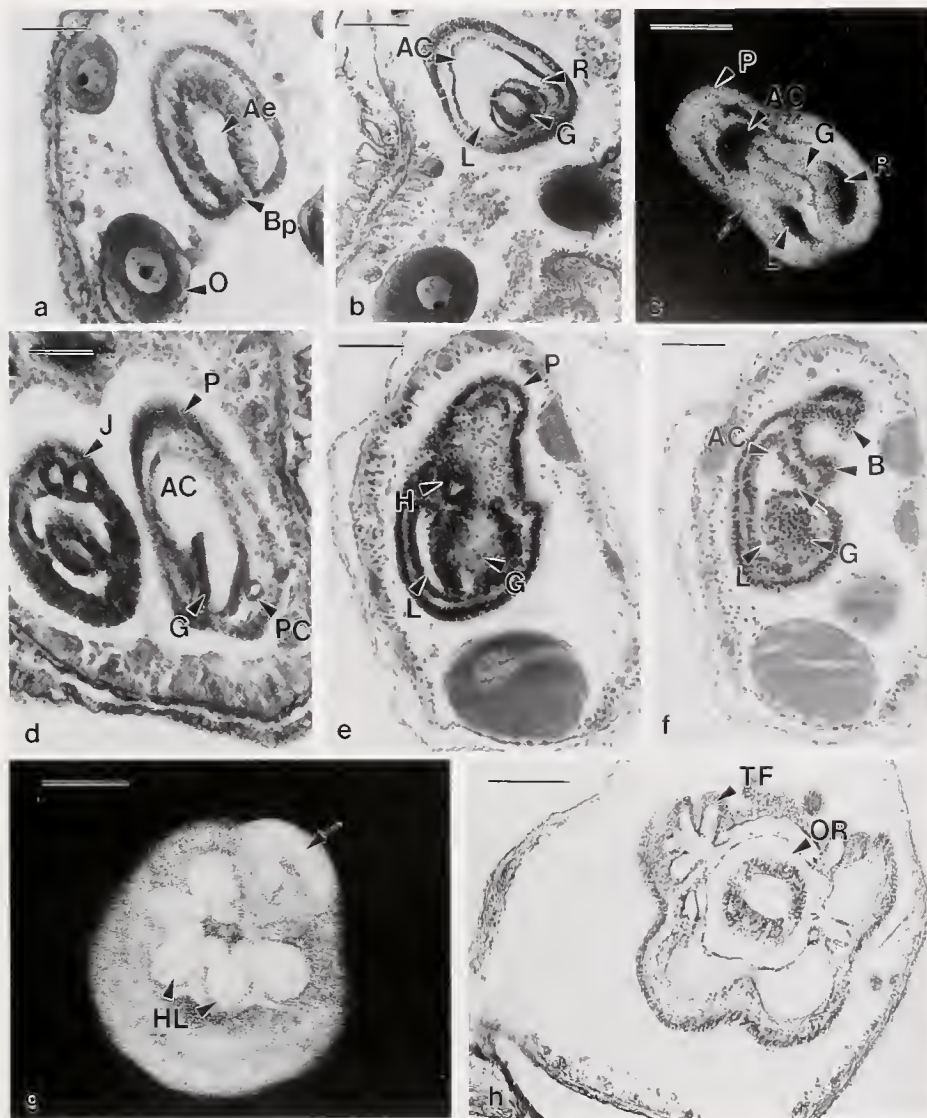


Figure 2. Light microscopy (Fig. 2a, b, d–f, h) and confocal microscopy (Fig. 2c, g) of internal development of *P. vivipara* (Fig. 2c, g) and *P. parvivipara* (Fig. 2a, b, d–f, h). Except in Fig. 2d, the larvae are orientated to facilitate presentation of right and left structures. Scale bars: Fig. 2a, b, d–f, h = 100 μ m; Fig. 2c, g = 50 μ m. (a) Gastrula with large archenteron (Ae) Bp, blastopore, O, oocyte. (b) Early brachiolaria with the large anterior coelom (AC) and left (L) and right (R) coeloms. G, gut. (c) Left side of a brachiolaria. The left (L) and right (R) posterior coeloms have separated from the anterior coelom (AC). The arrow indicates the position of the hydrocoel, the five lobes of which were seen in the adjacent optical section. G, gut, P, preoral lobe. (d) Section of a brachiolaria showing the small posterior enterocoel (PC). AC, anterior coelom; G, gut; J, metamorphosing juvenile; P, preoral lobe. (e) Tangential section of a brachiolaria showing the hydrocoel (H), left posterior coelom (L) and gut (G). P, preoral lobe. (f) Grazing section through the brachiolaria in Figure 2e showing the extension (arrow) of the anterior coelom (AC) into the left brachium. B, brachia; G, gut; L, left coelom. (g) Metamorphosing brachiolaria with the resorbing larval body (arrow) between hydrocoel lobes (HL) one and five. (h) Newly metamorphosed juvenile with two pairs of tube feet (TF). OR, oral water ring.

address numerous hypotheses on life history evolution (Byrne, 1995). Most importantly, molecular data confirm that *Patiriella* is a monophyletic taxon (Hart, Byrne, and Smith, unpub.) and so the comparisons made here and elsewhere (Byrne, 1991, 1992, 1995; Byrne and

Anderson, 1994; Cerra and Byrne, 1995a, b) are not confounded by divergent phylogeny.

In this investigation, the intragonadal development of *P. vivipara* and *P. parvivipara* is documented in detail. The larvae of these species are compared with those

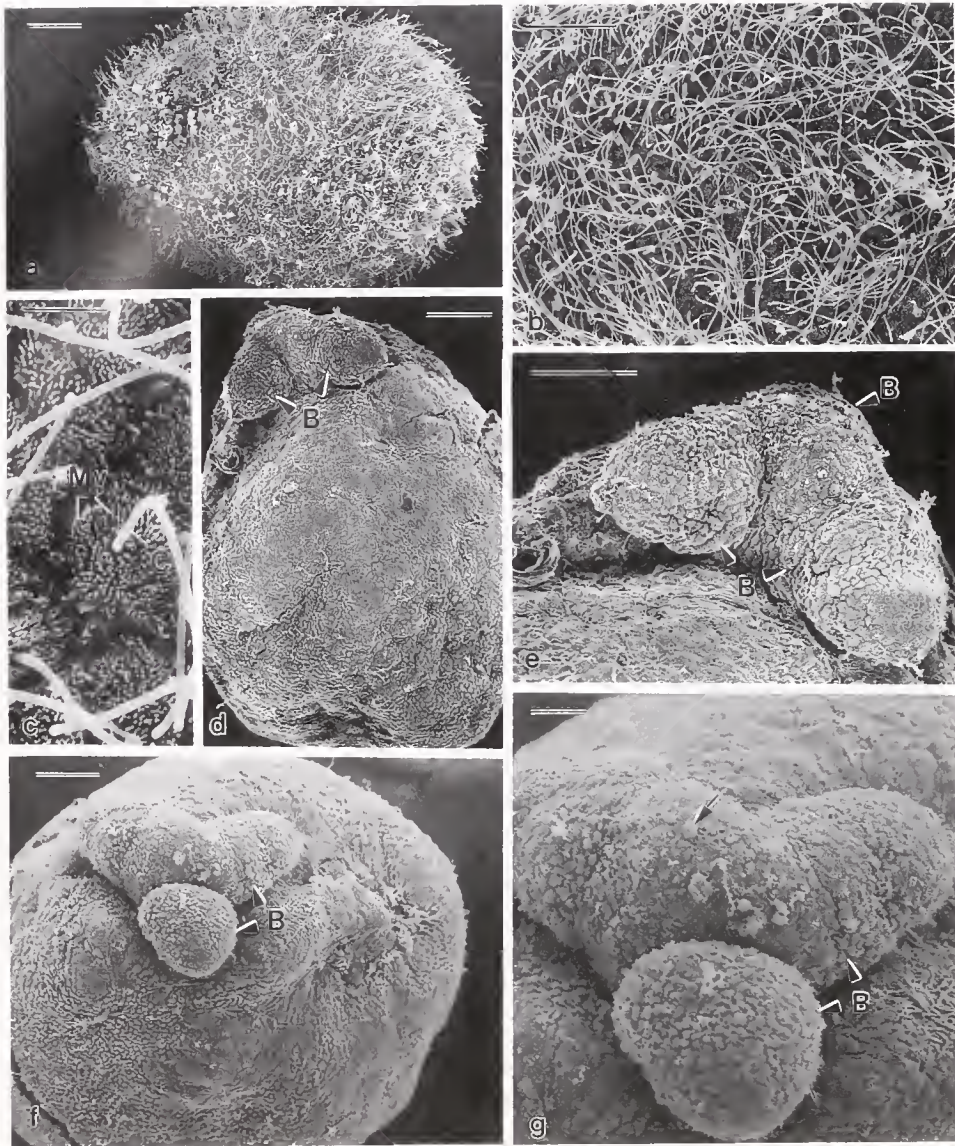


Figure 3. Scanning electron microscopy of pre-metamorphic development of *Patiriella vivipara* (Fig. 3b–g) and *P. parvivipara* (Fig. 3a). Scale bars: Fig. 3a, e = 20 μ m; Fig. 3b, g = 10 μ m; Fig. 3c = 2 μ m; Fig. 3d, f = 30 μ m (a) Slightly elongate gastrula covered by cilia. (b) The cilia are evenly distributed across the epithelial surface. (c) Detail of the epithelial cells showing cilia (C) surrounded by microvilli (Mv). (d) Side view of a late brachiolaria. B, brachia. (e) Detail of the brachiolar complex. The third brachium (B) is just visible (top) on the other side of the larva. (f) Anterior view of a late brachiolaria. B, brachia. (g) Detail of the brachiolar complex showing the three brachia (B). The area where an attachment disc would be expected (arrow) is unspecialized.

of the other *Patiriella* species to assess the changes in larval form associated with the evolution of viviparity (Byrne, 1991; Byrne and Barker, 1991; Byrne and Anderson, 1994). Particular attention was paid to the presence of vestigial features in the larvae and, considering the small size of the eggs, it was of interest to determine whether development is of the feeding or nonfeeding type.

Materials and Methods

Specimens of *Patiriella vivipara* were collected in Tasmania from Midway Point (42°48' S; 147°32' E) and the Tessellated Pavement (43°31' S; 147°56' E). *P. parvivipara* was collected from Cape Vivonne (34°44' S; 135°52' E), South Australia. The developmental stages of these species were isolated by dissection of the gonads.

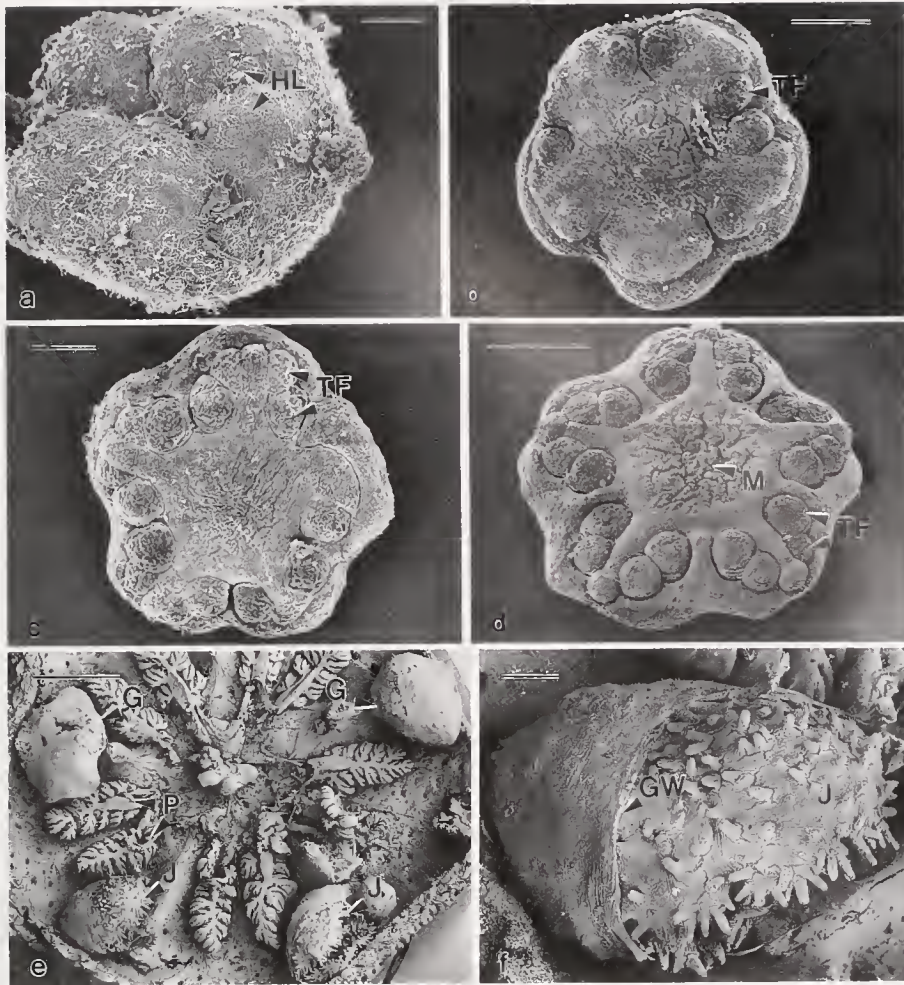


Figure 4. Scanning electron microscopy of metamorphosing juveniles and post-metamorphic development of *Patiriella vivipara* (Fig. 4a) and *P. parvivipara* (Fig. 4b–f). Scale bars: Fig. 4a = 30 μ m; Fig. 4b, c = 40 μ m; Fig. 4d = 100 μ m; Fig. 4e = 20 μ m; Fig. 4f = 300 μ m (a) Metamorphosing juvenile with the larval body fully resorbed and the hydrocoel lobes (HL) developing on the oral side. (b) Juvenile with first pairs of tube feet (TF) forming. (c) Juvenile with second pair of tube feet (TF) forming. (d) Metamorphosed juvenile with developing mouth (M) opening. TF, tube feet. (e) Dissected adult showing advanced juveniles (J) developing in the gonads (G). P, pyloric caeca. (f) As the juveniles grow the gonad wall (GW) stretches to accommodate them.

The size of the oocytes and the intragonadal young were measured with an ocular micrometer. The larvae were measured along their length and the juveniles were measured across their maximum radius (R), from the center of the disc to the tip of one arm.

Development of *Patiriella vivipara* and *P. parvivipara* was documented by light microscopy (LM), confocal microscopy, and scanning electron microscopy (SEM). Live specimens were photographed with a photomicroscope. For histology, gonads or whole sea stars were fixed in Bouin's fluid or 2.5% glutaraldehyde in seawater. Following fixation, the tissues were dehydrated in graded ethanols, embedded in paraffin, and sectioned (6 μ m

thick). Sections were stained with hematoxylin and eosin. For SEM, dissected embryos were fixed in 2.5% glutaraldehyde in filtered seawater for 1 h at room temperature. Following primary fixation, the specimens were washed in 2.5% NaHCO_3 (pH 7.2) and post-fixed in 2% OsO_4 in 1.25% NaHCO_3 for 1 h at room temperature. The specimens were then washed in distilled water and dehydrated in a graded series of ethanols, critical-point dried, sputter coated, and viewed with a JEOL JSM-35C scanning electron microscope.

For confocal microscopy, the larvae were fixed in 2% paraformaldehyde in seawater. The larvae were then dehydrated in graded ethanols, cleared in Histo-Clear (Na-

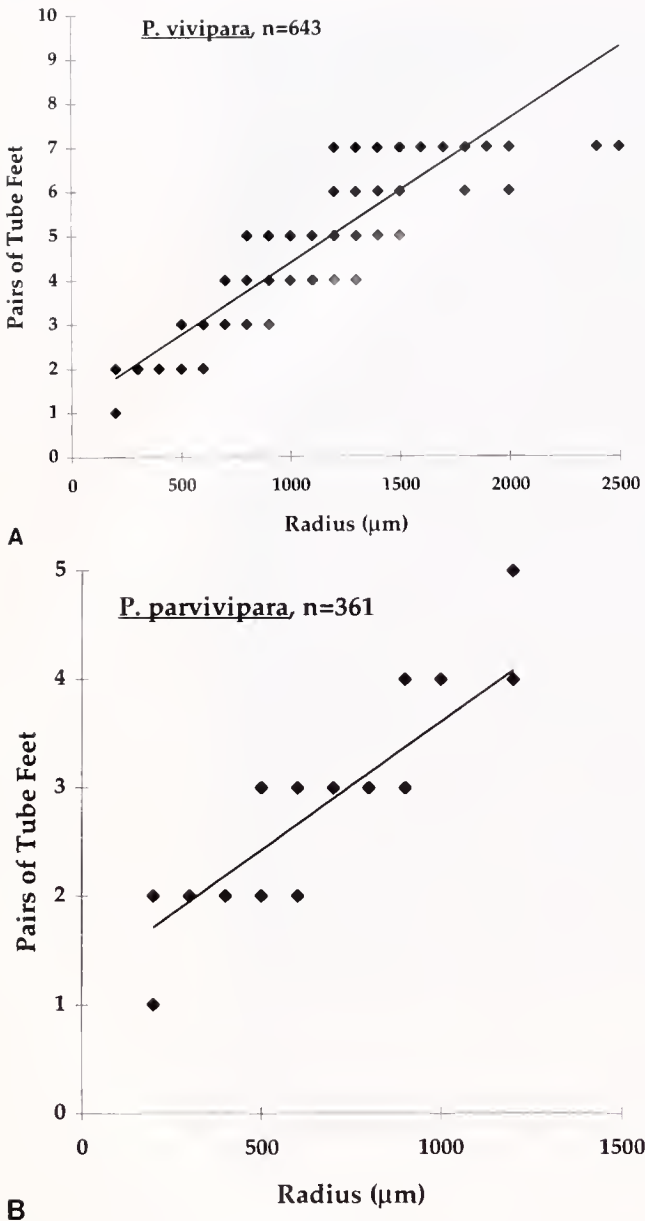


Figure 5. The relationship between the size of the intragonadal juveniles and the number of tube feet. (A) *Patiriella vivipara*, $r^2 = 0.845$. (B) *P. parvivipara* $r^2 = 0.710$. R = radius.

tional Diagnostics; Atlanta, Georgia), and mounted on slides. Optical sections of the specimens were made with a BIO-RAD MRC600 confocal microscope.

Development of the skeleton was documented by utilizing the birefringent properties of the calcite ossicles. The larvae are opaque and whole mounts of early larvae fixed in glutaraldehyde, dehydrated in alcohol, and cleared in Histo-Clear were observed with polarized light.

The sizes of the brachiolaria and newly metamorphosed juveniles of *P. vivipara* and *P. parvivipara* were

compared with these developmental stages in other *Patiriella* species. For *P. regularis*, *P. exigua*, and *P. calcar*, live larvae were measured with an ocular micrometer. For *P. gunnii*, measurements were made from photographs of live larvae. Measurements of newly metamorphosed *P. exigua* were made from live specimens. Measurements of newly metamorphosed *P. calcar* and *P. regularis* were made from preserved specimens.

Results

Pre-metamorphic development

Patiriella vivipara and *P. parvivipara* have the same pattern of development. Except where noted, the following description applies to both species.

The largest eggs encountered in the ovotestes of *P. vivipara* were pale orange primary oocytes and ova. These eggs had a mean diameter of $148 \mu\text{m}$ ($\text{SE} = 0.7$, $n = 21$). Ova were not encountered in the gonads of *P. parvivipara*. For this species, the largest eggs seen were $135 \mu\text{m}$ diameter ($n = 2$).

When the gonad was torn during dissection, minute gastrulae and brachiolaria larvae were released and swam in the seawater in the dissection dish (Fig. 1a, b). Due to the intragonadal location of the embryos, they were often surrounded by gametes (Fig. 2a, b, e, f). Development proceeded through the wrinkled blastula and gastrula stages. The gastrulae were uniformly ciliated and swam, propelled by the cilia, in the gonadal fluid (Figs. 2a, 3a–c). Each epithelial cell had one cilium surrounded by a field of microvilli (Fig. 3c). At no stage in development were the embryos attached to the gonadal wall. The gastrulae were round to elongate (Figs. 2a, 3a). This stage in *P. vivipara* had a mean length of $183 \mu\text{m}$ ($\text{SE} = 3.8$, $n = 5$) and in *P. parvivipara* had a mean length of $140 \mu\text{m}$ ($\text{SE} = 0$, $n = 5$). The blastopore closed as the gastrulae elongated.

The gastrulae gave rise to pear-shaped brachiolaria larvae that had a reduced brachiolar complex (Figs. 1a, b, 2c–f, 3d–g). Most of the larvae encountered had the simple pear-shaped profile seen in Fig. 2c. The larvae rotated as they swam with their anterior end forward. In *P. vivipara* these larvae had a mean length of $270 \mu\text{m}$ ($\text{SE} = 8.0$, $n = 17$), and in *P. parvivipara* they had a mean length of $207 \mu\text{m}$ ($\text{SE} = 1.9$, $n = 12$). The anterior preoral lobe of these larvae corresponds to the central brachiolar arm or brachium of planktonic brachiolariae. Two lateral extensions of the body developed at the base of the preoral lobe to form the right and left brachia, thereby completing the brachiolar complex. The area at the base of the arms, where an attachment disc would be expected to form, remained unspecialized (Fig. 3f, g). In *P. vivipara* and *P. parvivipara*, the brachiolar complex does not function as a larval attachment organ.

Internal development involved formation of one large enterocoel at the anterior end of the archenteron and one small posterior enterocoel on the left side of the archenteron (Fig. 2b–d). The anterior coelom extended into the preoral lobe and gave rise to the left and right coeloms on either side of the body (Fig. 2b, c, e, f). This coelom also gave rise to two short coeloms that extended into the lateral brachia (Fig. 2f). The hydrocoel developed on the left side of the larva soon after the brachiolaria stage was reached (Fig. 2c, e).

The brachiolaria metamorphosed in the gonadal lumen with formation of the juvenile at the rounded posterior end and reduction of the preoral lobe (Figs. 1b, 2g). On scanning view, the preoral lobe was composed of three small brachia (Fig. 3d–g). The ciliary cover was reduced (Fig. 3d, f). Metamorphosing larvae tended to spin around in one place. As the hydrocoel developed, the larval body was resorbed between hydrocoel lobes 1 and 5 (Fig. 2g). The rudiments of the adult skeleton appeared as birefringent spicules in metamorphosing larvae at a length of about 200 μm .

The rudiments of the adult arms and developing tube feet were evident on the oral side of metamorphosing juveniles (Figs. 1c, 2h, 4a–d). Metamorphosing larvae and newly metamorphosed juveniles had a mean diameter of 314 μm (SE = 21.5, n = 12) in *P. vivipara* and 244 μm (SE = 9.3, n = 37) in *P. parvivipara*. These larvae and newly metamorphosed stars were pale yellow. The mouth opened after the second pair of tube feet developed at about 400 μm diameter (Figs. 2h, 4d).

Post-metamorphic growth

Post-metamorphic development involved an increase in size of the juvenile and the addition of pairs of tube feet. The relationship between the number of tube feet and size of the juveniles is shown in Figure 5A, B. The largest intragonadal juvenile of *P. vivipara* had a radius of 2.5 mm and had 8 pairs of tube feet. In *P. parvivipara*, the largest intragonadal juvenile had a radius of 1.2 mm and 5 pairs of tube feet. The gonad wall stretched to accommodate growth of the juveniles (Fig. 4e, f).

Discussion

The presence of vestigial structures provides important evidence for elucidating the direction of evolutionary change (Strathmann, 1993). In *Patiriella vivipara* and *P. parvivipara*, the brachiolar complex is a vestigial remnant of the complex present in the other *Patiriella* species that use it for benthic attachment during metamorphosis. Metamorphosis in the *P. vivipara* and *P. parvivipara* does not involve attachment, and so the brachiolar complex is correspondingly reduced and nonfunctional. Not surprisingly, there is no trace of a plank-

trophic bipinnaria larva with the larval gut forming a closed structure that serves as a rudiment for the adult gut. The possession of a vestigial brachiolar complex and lecithotrophic development indicates that *P. vivipara* and *P. parvivipara* had a free-living lecithotrophic brachiolaria in their ancestry. The highly modified ontogenies of these species support the contention that viviparity is a derived life history positioned at the extreme end of the broadcasting–brooding continuum of life histories seen in *Patiriella* (Fig. 6).

With their central brachium and two lateral brachia, the larvae of *P. vivipara* and *P. parvivipara* are particularly similar to the early brachiolaria of the other *Patiriella* with lecithotrophic development (Byrne, 1995). In contrast to the viviparous species, however, brachiolar growth in the planktonic lecithotrophs involves further development of the arms, with the central brachium forming a conspicuous ventral curvature (Fig. 6; Byrne, 1991; Byrne and Anderson, 1994). Brachiolar growth in the benthic lecithotroph *P. exigua* results in formation of a tripod-shaped larva due to hypertrophic growth of the lateral brachia (Fig. 6; Byrne, 1995).

Pre-metamorphic development in *P. vivipara* and *P. parvivipara* appears to be largely supported by the vitellogenic reserves present in the egg. Although these reserves may be augmented by nutrients present in the gonadal fluid, the small size of the larvae indicates that dependence on extraembryonic nutrition is minimal. Unlike other asteroids with lecithotrophic development, including several *Patiriella* species, *P. vivipara* and *P. parvivipara* have a minute egg (Table 1, Fig. 6). As a result, the larvae and newly metamorphosed juveniles of these viviparous species are considerably smaller than those of their lecithotrophic congeners (Table 1). In contrast, the recruiting juveniles are the largest reported for the Asteroidea. Rather than channeling vitellogenic reserves into making large eggs, *P. vivipara* and *P. parvivipara* use cannibalism to support development at the post-metamorphic stage (Byrne, 1996). Parental investment by *P. vivipara* and *P. parvivipara* clearly favors the post-metamorphic progeny that may be incubated in the gonads for up to one year (Byrne, 1996).

Viviparity is rare in the Asteroidea. The only other asteroid known to have intragonadal development is another asterinid species, *Asterina pseudoexigua pacifica* (Table II, Komatsu *et al.*, 1990). Like *Patiriella vivipara* and *P. parvivipara*, the larvae of *A. pseudoexigua pacifica* are lecithotrophic (Komatsu *et al.*, 1990). In contrast to the minute brachiolaria of *P. vivipara* and *P. parvivipara*, the intragonadal brachiolaria of *A. pseudoexigua pacifica* is similar in size and form to planktonic lecithotrophic brachiolaria, and its development is supported by nutrients present in a large egg (450 μm diameter) (Komatsu *et al.*, 1990).

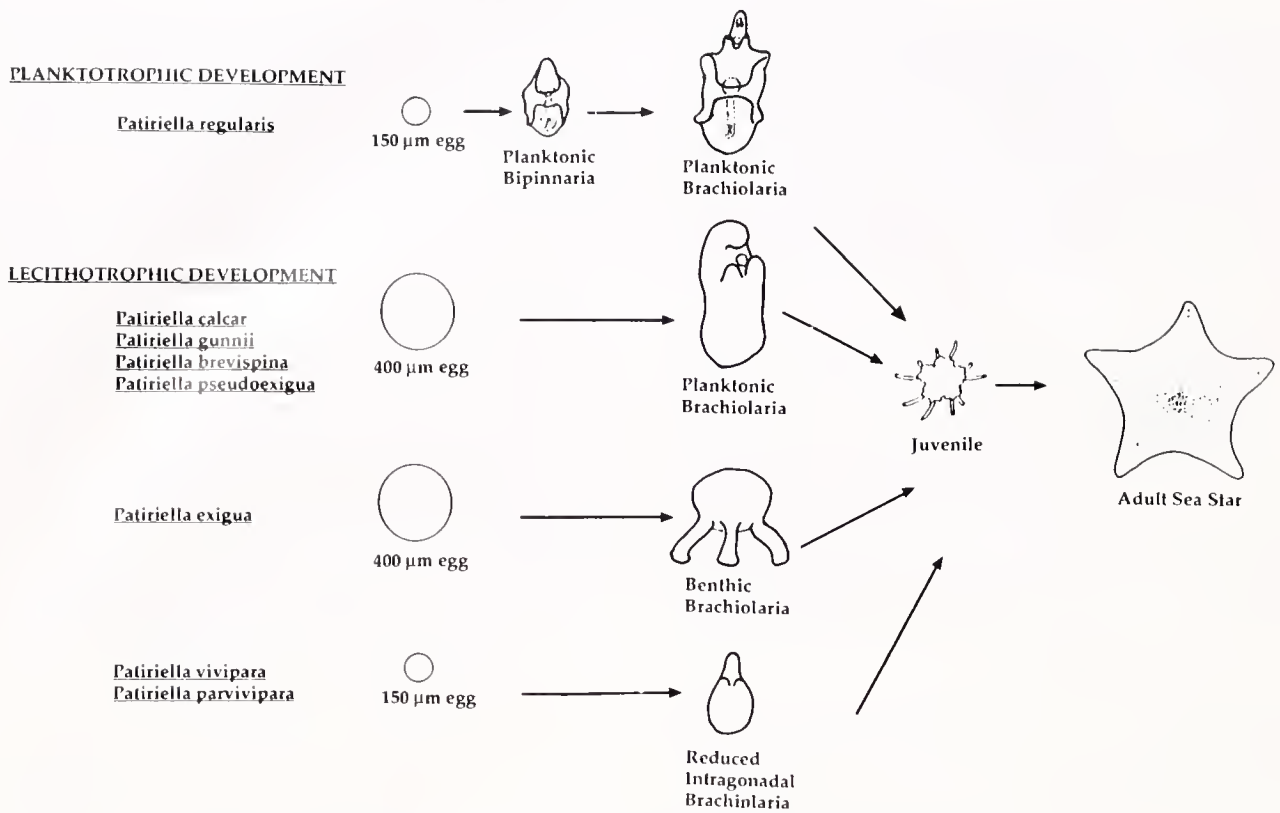


Figure 6. Diagram illustrating the developmental patterns of *Patiriella* species with the development of *P. regularis* (top) presumed to represent the ancestral state. Viviparity in *P. vivipara* and *P. parvivipara* is suggested to have arisen *via* an intermediate ancestor with benthic lecithotrophy. The evolutionary changes in developmental pattern are suggested to be from planktotrophy to planktonic lecithotrophy to benthic lecithotrophy to viviparity.

P. vivipara and *P. parvivipara* are unique among lecithotrophic asteroids in having a small egg. This prompts the question as to whether the evolution of viviparity in these species involved the retention of a small egg in the gonads for fertilization or retention of a large egg with a secondary reduction in egg size. Insights into this question are provided by systematic evidence and mtDNA sequence data (Dartnall, 1969, 1971; Keough and Dartnall, 1978; Hart, Byrne, and Smith, unpub.). On the basis of adult morphology, systematists place *P. vivipara* and *P. parvivipara* in the 'exigua' group and suggest that they were derived from an *exigua*-like ancestor (Dartnall, 1971; Keough and Dartnall, 1978). Distinguishing the species in this group depends on life-history characters and in archived collections often requires examination of the gonads for brooded young (Keough and Dartnall, 1978). Molecular data support inclusion of *P. vivipara* and *P. parvivipara* in the 'exigua' group and indicate that the viviparous *Patiriella* species diverged from an *exigua*-like ancestor approximately 3 million years ago (Hart, Byrne, and Smith, unpub.).

P. exigua deposits large eggs onto the substratum and abandons the developing embryos (Byrne, 1995). It has been suggested that the benthic life history of *P. exigua* may have set the stage for the evolution of intragonadal brooding in *Patiriella* (Byrne, 1991, 1995). Deposition of egg masses would have made the evolution of external brooding a relatively simple matter of the adult remaining with the egg mass, as exemplified by several closely related *Asterina* species (Table II, Strathmann *et al.*, 1984). In the proposed sequence of events it is suggested that intragonadal brooding arose from retention of eggs by an external brooder as occasionally occurs in *A. physalctica* (Strathmann *et al.*, 1984). We suggest that the transition to viviparity in *Patiriella* was from benthic lecithotrophy to intragonadal brooding as outlined in Figure 6. This transition would have involved retention of a large ovum for internal fertilization followed by a secondary reduction in egg size. This secondary reduction would have occurred in parallel with the evolution of intragonadal cannibalism to ensure that the juveniles emerge at a large size. The intragonadal location of the

Table II

Developmental patterns and larval types in the Family Asterinidae

Genus	Species	Spawning/Fertilization	Developmental pattern	Larval type (s)
<i>Patiriella</i>	<i>P. regularis</i>	Broadcaster	Planktotrophy	Bipinnaria and brachiolaria
	<i>P. calcar</i>	Broadcaster	Planktonic lecithotrophy	Brachiolaria
	<i>P. gunnii</i>	Broadcaster	Planktonic lecithotrophy	Brachiolaria
	<i>P. brevispina</i>	Broadcaster	Planktonic lecithotrophy	Brachiolaria
	<i>P. pseudoxigua</i>	Broadcaster	Planktonic lecithotrophy	Brachiolaria
	<i>P. exigua</i>	Deposits egg masses	Benthic lecithotrophy	Tripod brachiolaria
	<i>P. vivipara</i>	Intragonadal fertilization	Viviparous lecithotrophy	Reduced brachiolaria
	<i>P. parvivipara</i>	Intragonadal fertilization	Viviparous lecithotrophy	Reduced brachiolaria
<i>Asterina</i>	<i>A. pectinifera</i>	Broadcaster	Planktotrophy	Bipinnaria and brachiolaria
	<i>A. miniata</i>	Broadcaster	Planktotrophy	Bipinnaria and brachiolaria
	<i>A. batheri</i>	Broadcaster	Planktonic lecithotrophy	Brachiolaria
	<i>A. burtoni</i>	Broadcaster	Planktonic lecithotrophy	Brachiolaria
	<i>A. coronata japonica</i>	Broadcaster	Planktonic lecithotrophy	Brachiolaria
	<i>A. minor</i>	Deposits egg masses	Benthic lecithotrophy	Tripod brachiolaria
	<i>A. gibbosa</i>	Deposits egg masses	Benthic lecithotrophy	Brachiolaria with a foot-like attachment complex
	<i>A. phylactica</i>	Broods egg masses	Benthic lecithotrophy	Brachiolaria with a foot-like attachment complex
	<i>A. atypoida</i>	Broods egg masses	Benthic lecithotrophy	—
	<i>A. scobinata</i>	Broods egg masses	Benthic lecithotrophy	—
	<i>A. pseudoexigua pacifica</i>	Intragonadal fertilization	Viviparous lecithotrophy	Brachiolaria

Data from MacBride, 1896; Dartnall, 1970; James, 1972; Komatsu, 1975; Kano and Komatsu, 1978; Komatsu *et al.*, 1979; Marthy, 1980; Strathmann, 1987; Byrne, 1991, 1992, 1995, 1996; Chen and Chen, 1992; Chia *et al.*, 1993.

larvae would have released them from selective pressures to maintain the brachiolar complex, resulting in the simplification of the larval form.

The influence that developmental evolution has exerted on speciation in *Patiriella* is exemplified by *P. vivipara* and *P. parvivipara*. The morphological overlap between the phenotype and ecologies of adult *P. vivipara*, *P. parvivipara*, and *P. exigua* contrasts with the different phenotypes and ecologies of their larvae (Fig. 6). This contrast indicates that selection on the developmental stages has played an important role in the divergence of these three species. It also supports the suggestion of a decoupling of selective pressures on the adult and larval stages of *Patiriella* such that adult and larval stages respond differently to selective pressures during their evolution (Byrne and Anderson, 1994). This phenomenon is undoubtedly of great importance in taxa such as echinoderms which have complex life histories (Raff, 1992; Byrne and Anderson, 1994; Wray, 1995). With the divergence time between the viviparous species and *P. exigua* estimated to be 3 million years, it appears that evolution of viviparity was relatively rapid (Hart, Byrne, and Smith, unpub.). Rapid evolution of development is also reported for the sea urchin genus *Heliocidaris* (Smith *et al.*, 1990; Wray, 1995).

The extremely limited distributions of *P. vivipara* and *P. parvivipara*, together with their extended brood care,

indicate that their ranges are unlikely to increase and that they may be heading towards extinction. The distribution of *P. vivipara*, with two of the populations on either side of a 60-km-long peninsula, suggests that the present distribution of this species is a remnant of a previously wider range. Considering the important insights on life-history evolution that can be obtained through investigation of these unusual species, it is fortunate that they are extant. This scientific importance highlights *P. vivipara* and *P. parvivipara* as candidates for conservation.

Although it is not known whether brooding has imparted a selective advantage (or disadvantage) for *P. vivipara* and *P. parvivipara*, comparative embryology and systematic evidence suggest that evolution of viviparity was central to their divergence. The systematic distribution of brooding and other patterns of modified development appears to be skewed to certain echinoderm clades (Emlet, 1990; Pearse and Bosch, 1994). In the Asteroidea, the Family Asterinidae is a particularly illustrative example (Table II). In addition to *Patiriella*, the genus *Asterina* contains species with life histories that cover the broadcast-brooding continuum of life histories seen in the Asteroidea (Table II). Like *Patiriella*, *Asterina* species have developmental patterns ranging from planktotrophy to viviparity (Table II). There are also several fissiparous *Asterina* species (Achituv, 1969; Marsh, 1977). The developmental diversity seen in *Patiriella* and *Ast-*

erina is atypical of asteroid genera and supports the contention that the evolution of modified patterns of development in the Asteroidea and other echinoderms follows phylogenetic lineages (Pearse and Bosch, 1994).

Acknowledgments

Special thanks to G. Prestedge, M. O'Loughlin, and M. and H. Sowden for assistance with field collections. The Director and Mr. C. Proctor of the CSIRO Marine Laboratory in Hobart are thanked for use of facilities and assistance. Thanks also to G. Moreno for providing juveniles for measurement, R. Smith, C. Jeffrey, and the staff of the Electron Microscope Unit of the University of Sydney provided technical assistance. This work was supported by a grant from the Australian Research Council.

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Serotonergic Ligands Induce Spawning but not Oocyte Maturation in the Bivalve *Macra chinensis* from Central Japan

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Abstract. We examined the spawning sensitivity to serotonin and serotonergic ligands in the Japanese bivalve *Macra chinensis*. Spawning was induced by both injected and externally applied serotonin (5-hydroxytryptamine, 5-HT). The vertebrate 5-HT₂ receptor agonist alpha-methyl 5-HT and the selective 5HT_{1A} agonist 8-OH-DPAT were also effective at inducing spawning. However TFMPP (m-trifluoromethylphenylpiperazine, a vertebrate 5-HT₁ receptor agonist) and 1-methyl-chlorophenyl biguanide (a vertebrate 5-HT₃ agonist) were not effective spawning inducers. The 5-HT-induced spawning was blocked by mianserin (a vertebrate 5-HT₂ antagonist). The rank order of potency of the agonists was: 5-HT > alpha-methyl 5-HT > 8-OH-DPAT ≫ TFMPP > 1-methyl-chlorophenyl biguanide; these data support a growing body of literature invoking a mixed 5-HT₁/5-HT₂ pharmacological profile for serotonin receptors mediating reproductive processes in bivalves. However, neither 5-HT nor 8-OH-DPAT induced germinal vesicle breakdown (GVBD) in *Macra* oocytes. Sperm induced GVBD in a high percentage of oocytes. This is the first report of a bivalve in which spawning, but not GVBD, can be induced by 5-HT. This result might be expected because *Macra* spawns germinal vesicle oocytes that normally undergo GVBD upon fertilization, but is in contrast to the case of the closely related *Spisula* spp. in which serotonin induces both processes. The ability of 5-HT to induce spawning but not GVBD makes *Macra chinensis* a model organism for studying spawning and meiotic mechanisms in bivalves.

Introduction

The biogenic monoamine serotonin (5-hydroxytryptamine; 5-HT) is widely recognized as having salient neurohormonal effects on reproductive processes in bivalves including gamete release from gonad fragments (Matsutani and Nomura, 1982), spawning (Hirai *et al.*, 1988; Ram *et al.*, 1993), sperm reactivation (Kadam and Koide, 1990), parturition (Fong and Warner, 1995), and oocyte maturation (Kadam and Koide, 1989; Krantic *et al.*, 1991; Fong *et al.*, 1994; Gobet *et al.*, 1994; Deguchi and Osanai, 1995). Bivalves in the family Mactridae, especially *Spisula* spp., have provided especially good material for the study of the effect of serotonin on reproductive processes. Both spawning of prophase-I oocytes and the reinitiation of meiosis can be induced by 5-HT in *S. solidissima* and *S. sachalinensis* (Hirai *et al.*, 1988). Kadam and Koide (1989) tested various serotonergic ligands on oocyte maturation in *S. solidissima* and found that 8-OH-DPAT, a vertebrate 5-HT_{1A} agonist, significantly induced GVBD. Later Kadam *et al.* (1990) reported that both 8-OH-DPAT and alpha-methyl 5-HT, a 5-HT₂ agonist, stimulated uptake of ⁴⁵Ca²⁺. This uptake could be blocked by mianserin, a 5-HT₂ antagonist. More thorough pharmacological investigations of 5-HT-induced oocyte maturation in *S. solidissima* have been reported by Krantic *et al.* (1991).

Macra chinensis and *Spisula* spp. are closely related species in the bivalve family Mactridae. *M. chinensis* supports an important bivalve fishery in Japan (Sakurai *et al.*, 1992; Sakurai, 1993, 1994) and Korea (Chung *et al.*, 1987). As such, information on the physiological mechanisms regulating spawning and oocyte maturation in this species is important not only for aquacultural

purposes, but also to compare reproductive mechanisms in closely related species. We tested various serotonin analogs for effects on spawning and oocyte maturation in *M. chinensis*. Our experiments revealed that, as in *Spisula*, 5-HT and related compounds are potent inducers of spawning, but unlike *Spisula*, serotonergic compounds do not cause significant GVBD in *Macra*.

Materials and Methods

Animals

Mature *Macra chinensis* (4–6 cm shell length) were collected in May 1995 by fishermen in Tokyo Bay, Chiba Prefecture, and immediately shipped to the Asamushi Marine Biological Station. Animals were maintained in running seawater until used. All experiments were performed from 23 to 30 June 1995.

Spawning and GVBD assays

To induce spawning, animals were injected in the foot with 0.4 ml of 5-HT, 5-HT ligands, or natural seawater (SW) as a control. Immediately after injection, animals were placed in plastic containers containing 200 ml of SW, and observed for evidence of spawning. All spawning experiments lasted one hour, after which all non-spawners were injected with 5-HT (determined to be a good inducer of spawning after initial experiment) as a test for their ability to spawn.

The effect of mianserin, a vertebrate 5-HT₂ antagonist that inhibits a number of reproductive processes in marine and freshwater bivalves, was tested as follows. Various concentrations of antagonist were injected into the foot for 15–20 min before a subsequent injection of 5-HT combined with mianserin at the same concentration as in the first injection. Responses were monitored for 1 h after the second injection.

For the analysis of GVBD, prophase-I oocytes were dissected from ovaries or, on one occasion, obtained by a natural spawning. In both cases, released oocytes had large germinal vesicles that were clearly visible (Deguchi and Osanai, 1994). GV oocytes were pipetted into wells of a 24-well culture plate and subjected to various serotonergic agents, SW, or sperm from dissected testes. After 30–40 min, 70–211 oocytes were observed for germinal vesicle breakdown and the percent of the oocytes that had undergone GVBD was calculated.

Data for comparisons of percent spawning between treatments and controls were analyzed with Fisher's Exact Test (Zar, 1984).

Serotonin and mianserin were purchased from Sigma Chemical Co. (St. Louis, Missouri). All other ligands

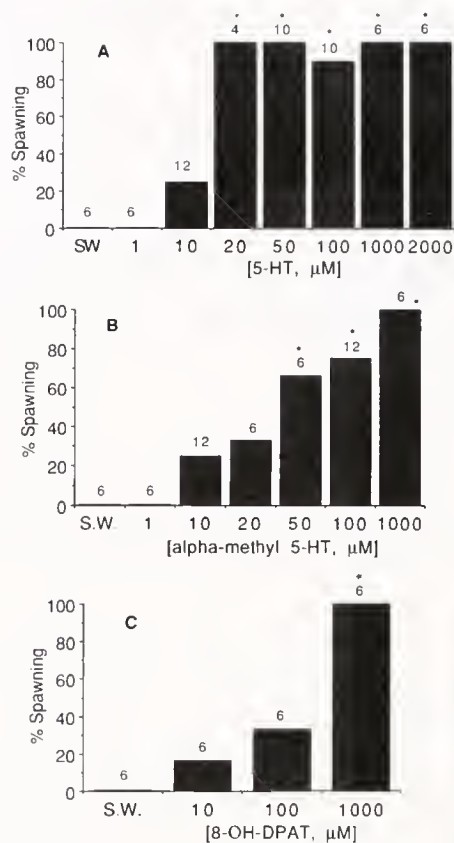


Figure 1. Percent spawning of *Macra chinensis* after injection of serotonergic compounds or vehicle control (seawater, SW). (A) Serotonin (5-HT). (B) Alpha-methyl-5-HT. (C) 8-OH-DPAT. Numbers of clams tested are given above bars. *: $P < 0.05$ compared to control (SW). Data from males ($n = 62$) and females ($n = 60$) have been combined as there was no significant difference in responses between sexes.

were obtained from either Research Biochemicals Inc. (Natick, Massachusetts) or Funakoshi Co. (Japan).

Results

Both male and female *Macra chinensis* responded strongly to injected 5-HT. Compared with the seawater control, 5-HT concentrations from 20 μM to 2 mM significantly induced spawning in 90–100% of clams tested (Fisher's Exact Test; $P < 0.004$ – 0.0001 ; Fig. 1A). In all cases, spawning took place in <3 min, and in 1 mM 5-HT, spawning was extremely rapid (mean time to spawning = 46.4 s for males; 43.0 s for females). Spawning oocytes had germinal vesicles and were fertilizable with spawned sperm. The 5-HT₂ agonist alpha-methyl 5-HT also significantly induced spawning in both males and females at concentrations from 50 μM to 1 mM (Fisher's Exact Test, $P < 0.03$ – 0.001 ; Fig. 1B). The 5-HT_{1A} agonist 8-OH-DPAT also stimulated

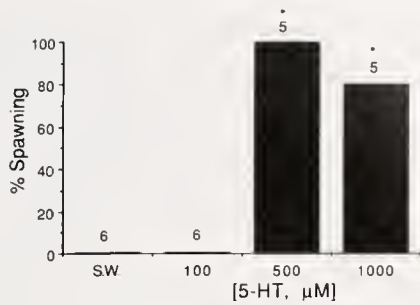


Figure 2. Percent spawning of *Mactra chinensis* after external application of different concentrations of 5-HT or seawater (SW). Numbers of clams tested are given above bars. *: $P < 0.05$ compared to control (SW).

spawning, but was not as potent as either 5-HT or alpha-methyl 5-HT; concentrations as high as 1 mM were required to produce significantly more spawning than controls (Fisher's Exact Test, $P < 0.001$; Fig. 1C). None of the 18 SW-injected controls spawned in the above series of experiments. Furthermore, neither the 5-HT₁ agonist TFMPP nor the 5-HT₃ agonist 1-methyl-chlorophenyl biguanide (1-m-c-b) were effective at inducing spawning. In TFMPP, only 4% (1 of 25) of the clams spawned, and in 1-m-c-b, none of the six clams tested spawned. External application of 5-HT (500 μ M and 1 mM) was sufficient to induce a high percentage of spawning (Fisher's Exact Test, $P < 0.002$ – 0.001 ; Fig. 2).

Although both 5-HT and alpha-methyl 5-HT were potent agonists, *Mactra* responded to 5-HT more rapidly. At 50 μ M and 100 μ M, significant (t -tests; $P < 0.05$ – 0.06) differences in time to spawning exist between clams in 5-HT and alpha-methyl 5-HT (Fig. 3).

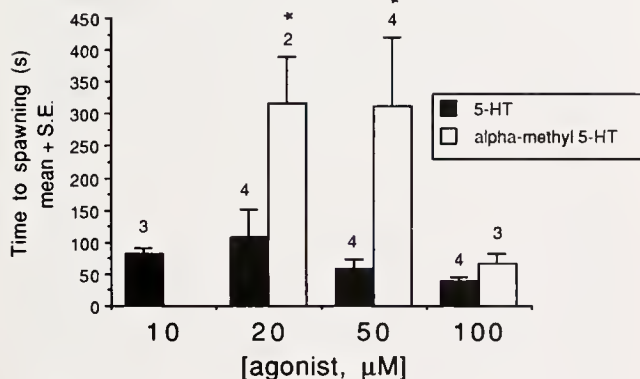


Figure 3. Time to spawning (mean + S.E.) of *Mactra chinensis* after injection of various concentrations of either 5-HT (filled bars) or alpha-methyl 5-HT (open bars). Numbers of clams that spawned are given above error bars. *: $P < 0.05$.

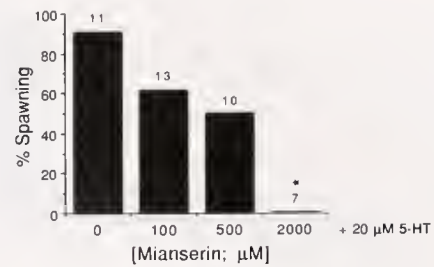


Figure 4. Effect of various concentrations of mianserin on spawning induced by 20 μ M 5-HT in *Mactra chinensis*. Clams were first injected with mianserin, then after 15–20 min, a second injection of 5-HT was given. Numbers of clams tested are given above bars. *: $P < 0.05$ compared to "no mianserin" control.

The specific 5-HT₂ antagonist mianserin effectively blocked, in a dose-dependent manner, the spawning induced by 5-HT (20 μ M) (Fig. 4). Pre-treatment of clams with mianserin (2 mM) significantly blocked spawning produced by the injection of 5-HT (Fisher's Exact Test, $P < 0.002$). Moreover, even though clams pre-treated with 500 μ M mianserin spawned after injection with 5-HT, some groups of clams took longer, on average, to spawn than control clams. Latency to spawning of clams pre-treated with 500 μ M mianserin and then injected with 5-HT took significantly longer to spawn than the 20 μ M 5-HT

Table 1

Summary of experiments with *Mactra chinensis* on the blockage of serotonin-induced spawning by mianserin, a 5-HT₂ antagonist

1st injection	2nd injection	# Spawmed/ total	Time (s) to spawning. Mean + (SEM)
20 μ M 5-HT		9/10	75.7 (7.6)
1 mM 5-HT		8/8	37.0 (4.2)
100 μ M mianserin		2/8	900.0 (12.5)
100 μ M mianserin	20 μ M 5-HT + 100 μ M mianserin	8/13	340.8 (210)
500 μ M mianserin		0/6	
500 μ M mianserin	20 μ M 5-HT + 500 μ M mianserin	5/10	125.8 (18.9)
500 μ M mianserin	1 mM 5-HT + 500 μ M mianserin	6/6	83.3 (27.1)
2 mM mianserin		0/6	
2 mM mianserin	20 μ M 5-HT + 2 mM mianserin	0/7	
2 mM mianserin	1 mM 5-HT + 2 mM mianserin	6/6	110.8 (73.9)

In cases where two injections were made, the second injection was given 15–20 min after the first injection. 5-HT: Serotonin.

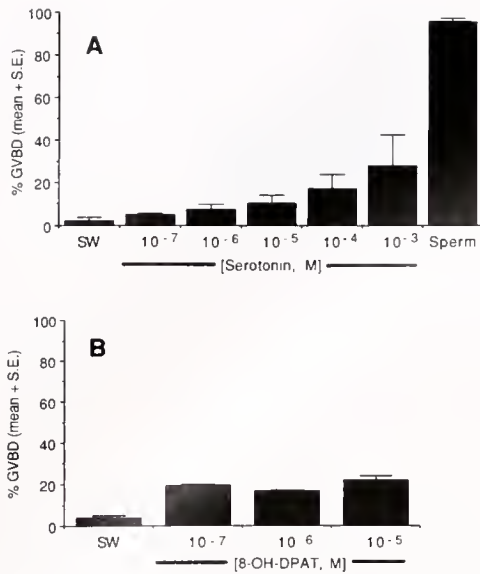


Figure 5. Percent GVBD (mean $\pm$ S.E.) in *Mactra* oocytes treated with (A) 5-HT, or sperm as a positive control ($n = 6$ females) and (B) 8-OH-DPAT ($n = 3$ females). SW = seawater.

HT control (t -test, $t = 2.9$, $P < 0.01$), and the 1 mM 5-HT control (t -test, $t = 1.9$, $P < 0.07$; Table 1).

In contrast to spawning, oocyte maturation as measured by GVBD was not significantly induced by 5-HT or its ligands. Neither 5-HT nor 8-OH-DPAT induced GVBD in a high percentage of oocytes, whereas sperm induced GVBD in over 95% of the oocytes (Fig. 5A, B).

Discussion

Injection of 5-HT, alpha-methyl 5-HT, a 5-HT₂ agonist, and 8-OH-DPAT, a 5-HT_{1A} agonist all produced significant dose-dependent induction of spawning in *Mactra chinensis*. In addition, external application of 5-HT also induced spawning. However, the 5-HT₁ receptor agonist TFMPP and the 5-HT₃ receptor agonist 1-m-chlorophenyl biguanide had little or no effect on spawning. Serotonin was effective at inducing spawning at lower concentrations than both alpha-methyl 5-HT and 8-OH-DPAT, and clams injected with 5-HT spawned significantly faster than those injected with alpha-methyl 5-HT. Therefore, the rank order of potency for agonists was 5-HT > alpha-methyl 5-HT > 8-OH-DPAT $\gg$ TFMPP > 1-methyl-chlorophenyl biguanide. These experiments support a growing body of literature indicating that 5-HT receptors mediating reproductive processes in bivalves show a mixed 5-HT₁/5-HT₂ pharmacological profile, unlike any described for vertebrates (e.g., Kadam *et al.*, 1991; Krantic *et al.*, 1991; Fong *et al.*, 1993; Gobet *et al.*, 1994).

In bivalve molluscs, all oocytes are initially arrested at prophase-I within the ovary. At the time of spawning, oocytes are released either at prophase-I or metaphase-I. In species such as *Mytilus edulis*, *Dreissena polymorpha*, and *Ruditapes philippinarum*, prophase-I-arrested oocytes reinitiate meiosis (including GVBD) under the influence of neurohormones such as 5-HT. Such oocytes are spawned at metaphase-I where they remain until fertilization. By contrast, species such as *Spisula solidissima*, *Barnea candida*, and *Mactra chinensis* spawn prophase-I arrested oocytes which, in nature, only reinitiate meiosis after fertilization. In the oyster *Crassostrea gigas*, both prophase-I and metaphase-I oocytes can be fertilized, although in nature the latter occurs.

Oocytes from *Spisula* spp. have provided good material for the study of oocyte maturation and other cell cycle processes (Hirai *et al.*, 1988; Kadam and Koide, 1989; Kadam *et al.*, 1990; Krantic *et al.*, 1991; Dube, 1992). In *Spisula*, both spawning and GVBD can be induced by 5-HT and 5-HT agonists. However, considering that *Spisula* spawns prophase-I oocytes, the fact that 5-HT can induce GVBD seems puzzling in a functional sense. The reported 5-HT concentrations necessary to induce GVBD in spawned oocytes would not be present freely in seawater. Thus any effect of 5-HT or related agents on GVBD may simply mimic that of sperm. However, Juneja *et al.* (1993) found that lower concentrations of 5-HT enhanced the fertilizability of *Spisula* oocytes, indicating a 5-HT mediated process that might occur in the *Spisula* gonad during spawning. Although functional 5-HT receptors have been reported in *Spisula* oocyte plasma membranes (Kadam *et al.*, 1991), and were initially named 5-HT₅ (Krantic *et al.*, 1993), Guerrier *et al.* (1993) considered these receptors evolutionary vestiges from an ancestor with metaphase-I-arrested oocytes. In our experiments, we found that 5-HT and 5-HT ligands induce spawning, but do not induce GVBD in a large percentage of dissected or naturally spawned oocytes of *Mactra chinensis*. In contrast and as expected, sperm induced a high percentage of oocytes to undergo GVBD. Thus, within the animal, serotonin may be released internally by nerve terminals to bind to receptors to initiate spawning; but spawned oocytes, which never encounter high concentrations of 5-HT, are not sensitive to it. This is the first report of a bivalve in which 5-HT can induce spawning, but not GVBD. Because spawned GV oocytes are not responsive to 5-HT, GVBD can be examined without first sensitizing the oocytes to 5-HT. Thus, *Mactra chinensis* may be a model organism for studying spawning and oocyte maturation mechanisms in bivalves.

Although the importance of serotonin as a neurohormone controlling reproductive processes in bivalves has

Table II

Reproductive processes induced by serotonin and serotonergic ligands in selected bivalve species

Species (Family)	Reproductive process	Serotonergic ligand	Reference
<i>Dreissena polymorpha</i> (Dreissenidae)	Spawning	5-HT, 8-OH-DPAT, TFMPP, 1-NP	Fong <i>et al.</i> , 1993
	GVBD	5-HT, 8-OH-DPAT	Fong <i>et al.</i> , 1994
<i>Ruditapes philippinarum</i> (Veneridae)	Spawning	5-HT	Osanai and Kuraishi, 1988
	GVBD	5-HT, 8-OH-DPAT, TFMPP	Gobet <i>et al.</i> , 1994; Osanai and Kuraishi, 1988
<i>Patinopecten yessoensis</i> (Pectinidae)	Release of oocytes from ovarian fragments	5-HT	Matsutani and Nomura, 1982
<i>Spisula solidissima</i> (Mactridae)	Spawning	5-HT	Hirai <i>et al.</i> , 1988
	GVBD	5-HT, 8-OH-DPAT	Hirai <i>et al.</i> , 1988
		alpha-methyl 5-HT	Krantic <i>et al.</i> , 1991
	Sperm motility	5-HT, 8-OH-DPAT, 5-methoxytryptamine	Kadam <i>et al.</i> , 1991 Kadam and Koide, 1990
<i>Crassostrea gigas</i> (Ostreidae)	GVBD	5-HT	Osanai and Kuraishi, 1988
<i>Hiatella flaccida</i> (Hiatellidae)	GVBD	5-HT	Togo <i>et al.</i> , 1993
<i>Potamocorbula amurensis</i> (Corbulidae)	Spawning	5-HT, 8-OH-DPAT	Pers. obs.
<i>Sphaerium transversum</i> (Sphaeriidae)	Parturition	5-HT, alpha-methyl 5-HT	Fong and Warner, 1995 unpub. data

5-HT = 5-Hydroxytryptamine, 8-OH-DPAT = 8-Hydroxydiethylaminotetralin hydrobromide, TFMPP = m-trifluoromethylphenylpiperazine, 1-NP = 1-(1-naphthyl)piperazine.

been well established, the number of species known to respond to this biogenic amine and similar compounds is still quite small and is restricted mainly to economically important species (Table II). Characterization of 5-HT receptors has been reported in only a few bivalve species such as *Dreissena polymorpha* (Fong *et al.*, 1993) and *Spisula solidissima* (Krantic *et al.*, 1993). In the gastropod *Lymnaea stagnalis*, a gene for a G-protein coupled 5-HT receptor (5-HT₁lym) was cloned and expressed in COS-7 cells (Sugamori *et al.*, 1993). To our knowledge, however, no bivalve serotonin receptors have been cloned and sequenced; hence we know little about the structure or evolutionary history of these proteins. These and further studies on the reproductive effects of 5-HT on a diverse array of bivalve species are required if the evolution of bivalve and molluscan 5-HT receptors is to be better understood.

Acknowledgments

We thank Y. Yamada for supplying specimens of *Macra*. We also thank S. Krantic and two anonymous reviewers for commenting on the manuscript. Travel funds for P. F. were provided by the office of the provost of Gettysburg College.

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Subunit Composition of the Crustacean Hemocyanins: Divergence in Incipient Speciation

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Abstract. The monomeric subunit composition of the oxygen carrier hemocyanin was examined in samples of the *Sesarma reticulatum* complex and of *Uca minax*, both of which are believed to be in the process of speciation. The samples were taken on the Atlantic and Gulf of Mexico coasts, from disjunct populations that are believed to have been isolated by the Florida peninsula since the retreat of the last glacier. In Atlantic and Gulf samples of the *S. reticulatum* complex, which is believed to be in the terminal stages of speciation, the hemocyanins differed qualitatively. Several electrophoretic bands found in one group, including an invariant band, were totally absent from the other. This difference exceeds that reported in a previous investigation of a variety of polymorphic allozymes in this species complex. It also exceeds the physiologically labile differences in hemocyanins found previously within a panmictic species of brachyuran crustaceans. In *U. minax*, which is believed to have diverged less, Atlantic and Gulf animals expressed the same number of electrophoretic bands at exactly the same positions. Nonetheless, highly significant differences in band frequencies distinguished both Atlantic samples from the Gulf sample, and somewhat less significant differences distinguished the Atlantic cold temperate zone samples from the warm temperate zone ones. The phenotypes of the major bands, defined as those present in high densities, qualitatively distinguished Atlantic from Gulf animals, but they did not differentiate the two Atlantic samples. The difference between Atlantic and Gulf members of this species also exceeds that found previously among polymorphic allozymes. These findings further support the hypothesis

that the hemocyanins are among the first proteins to diverge structurally in brachyuran speciation.

Introduction

With few exceptions, the crustacean hemocyanins (Hcs) are composed of many different polypeptide chains. When separated by charge in native polyacrylamide electrophoresis (PAGE), a dozen or more Cu-containing bands have been reported in some species (Mangum and Greaves, 1995; Mangum, 1996). In most species investigated thus far, the unusual heterogeneity provides the basis for intraspecific polymorphisms of a magnitude that is unprecedented in the literature on allozyme variation (Mangum, 1990, 1993a, 1996; Callcott and Mangum, 1993; Mangum and Greaves, 1996). In spite of these polymorphisms, the PAGE banding pattern appears to be species specific, even in sibling and previously cryptic species (Reese and Mangum, 1994; Mangum, 1996). The specificity suggests that the oxygen carrier diverges early in speciation, a hypothesis that is consistent with the quantitative importance of crustacean Hcs in aerobic metabolism (Truchot, 1992).

If the hypothesis is correct, one might expect to find differences between populations that are in the process of speciation. Below the species level, however, the available information on structural divergence of the crustacean Hcs appears to be contradictory. On the one hand, hybrid lobsters produced in the laboratory by unnatural matings of sibling, allopatric parental species can be readily distinguished from both parents (Mangum, 1993b). Less convincingly, because the comparison is based on different procedures used in different laboratories, samples from two subspecies of a fiddler crab differed so much that no bands were clearly shared by the two (Mangum and Greaves, 1996). On the other hand, no difference was found between the stone crab *Menippe*

Received 23 October 1995; accepted 4 April 1996.

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mercenaria and the hybrid progeny of postulated natural matings between it and its sibling and former cryptor *M. adina* (Mangum, 1996). Also less convincing for the same reason described above, the phenotypes found in another pair of fiddler crab subspecies were quite similar (Mangum and Greaves, 1995).

In the present investigation, we analyzed the banding patterns of Hc monomers found in populations isolated by the Florida peninsula since the retreat of the last glacier. One pair is believed to be in the terminal stages of speciation, and the other group is believed to have diverged less. Because each sample proved to be polymorphic, we also characterized the intraspecific variation in detail, to ensure against confusing it with geographic divergence.

The species investigated are (1) the grapsid marsh crab *Sesarma reticulatum* (Say) from the Atlantic coast of North America and its sibling from the Gulf of Mexico, which has not yet been described and is currently known as *Sesarma* sp. (near *reticulatum*) (Zimmerman and Felder, 1991), and (2) the fiddler crab *Uca minax* (LeConte), also from both coasts.

Materials and Methods

Animals

All individuals investigated appeared to be intermolt crabs, as indicated by hard exoskeletons.

Atlantic populations of *Sesarma reticulatum* were sampled at Taskinas Creek and Indian Field Creek, both of which empty into the York River estuary in Virginia. Gulf of Mexico populations of *Sesarma* sp. were sampled at Cocodrie and Joseph's Harbor (Rockefeller refuge) in Louisiana, and at Ocean Springs in Mississippi. Atlantic cold temperate zone populations of *Uca minax* were sampled at five sites on the York River estuary (Ware Creek, Croaker Landing, York River State Park, Kings Creek, and Indian Field Creek) and one on the James River estuary (College Creek) in Virginia; these sampling sites are distributed along a salinity gradient ranging from oligohaline to upper mesohaline. A warm temperate zone population was sampled at Holden Beach in North Carolina. Gulf populations were sampled at Cypremort Point in Louisiana and Fort Bayou near Ocean Springs in Mississippi.

Preparation of material

Animals were bled immediately with a hypodermic syringe, and the blood was frozen until shortly before analysis. After thawing, serum was expressed from the clot by centrifugation, and an aliquot was diluted with a buffer (50 mmol l⁻¹ Tris HCl, pH 8.9, containing 10 mmol l⁻¹ EDTA) that dissociates Hc oligomers to

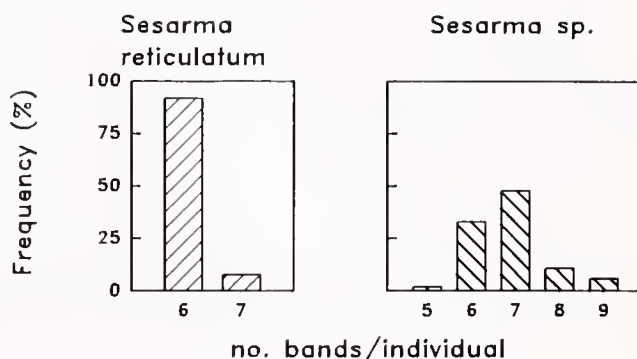


Figure 1. The frequency distribution of the total number of bands found in an individual of *Sesarma reticulatum* ($n = 97$) and *Sesarma* sp. ($n = 64$).

their monomers and thus minimizes light scattering. Hc concentration was estimated from the absorbance of monomers, using the extinction coefficient for *Cancer borealis* Hc (Nickerson and van Holde, 1971), and additional aliquots of material were then diluted with the same buffer to produce a concentration of about 5 $\mu\text{g } \mu\text{l}^{-1}$. We emphasize the importance of the dissociation step in this procedure, which, in our experience, eliminates an error of about 15%.

Electrophoresis

Native PAGE of dissociated material was performed at constant current, using the procedures and reagents detailed by Hames and Rickwood (1990). Two volumes (10 and 3 μl) of the material from each individual were examined. Most gels were stained only with Coomassie blue. To detect Cu, however, examples from each sample that collectively expressed all bands found in the sample were first electrophoresed, and then inspected on a long-wave UV transilluminator in the presence of bathocuproine sulfonate (Bruyninckx *et al.*, 1979). To estimate the proportions of the bands, examples were scanned with a BioRad Video Densitometer (Model 300A), and the scans were quantified with the 1D Analyst software package.

Results

Sesarma reticulatum complex

No difference was found between the individuals collected in Louisiana ($n = 61$) and Mississippi (3), and none was found between the individuals collected from the two tidal creeks in Virginia ($n = 97$). In both cases the data were combined for analysis and presentation. All bands were positive for Cu, and no band was related to sex.

Whereas all but a few individuals in the Atlantic sample expressed 6 Hc bands, the modal number in the Gulf

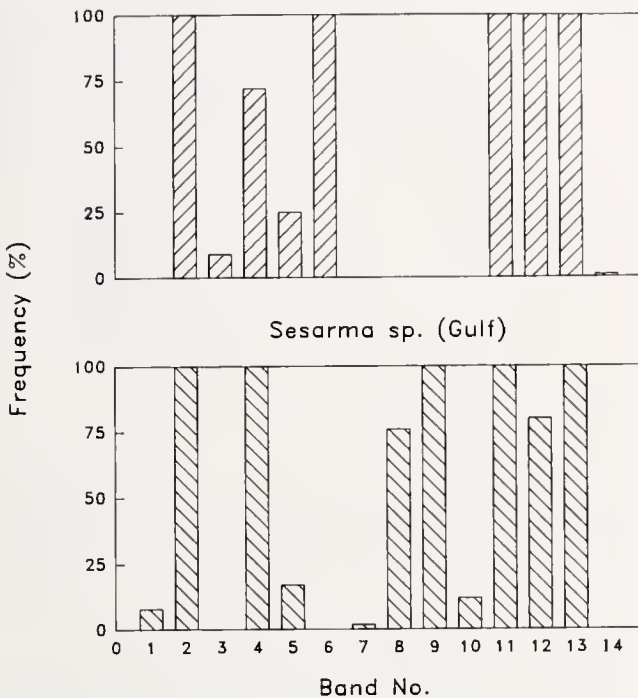
Sesarma reticulatum (Atlantic)

Figure 2. The frequencies of each band in *Sesarma reticulatum* and *Sesarma sp.* Sample size as in Fig. 1.

sample was 7 (Fig. 1). A total of only 9 bands was found in the Atlantic animals, but a total of 11 occurred in the Gulf animals (Figs. 2 and 3). Bands 2, 4, 5, and 11–13 in the two groups co-migrated to identical positions (Fig. 3). Two of these, however, differed in variability. Band 4

was invariant in *Sesarma sp.* but not in *S. reticulatum* (Fig. 2), in which it was often found in lower density as well (Fig. 3). Band 12 was variable in *Sesarma sp.* but not in *S. reticulatum* (Fig. 2).

In both groups a cathodic cluster of bands separated at some distance from an anodic cluster (Fig. 3). In both groups, the most common phenotype included bands in each cluster that co-migrated with bands in the other group, namely bands 2, 4, and 11–13. Nonetheless, the two groups were reliably distinguished by the presence in *Sesarma sp.* of a pair of both frequent and dense bands (8 and 9) in between the two clusters; because it was invariant, band 9 was an absolute marker. Members of this pair of bands were never found in *S. reticulatum* (Figs. 2 and 3). Additional, though less striking, differences include (1) one or two cathodic bands in each group that were not found in the other (1 in *Sesarma sp.* and 6 in *S. reticulatum*), and (2) two infrequent and less dense members (7 and 10) of the set of bands in between the cathodic and anodic clusters in *Sesarma sp.* Band 14 was found only once and only in trace densities in *Sesarma reticulatum*; it is unlikely to be a useful marker.

Both groups were polymorphic: 7 phenotypes were found in *S. reticulatum*, and 13 were found in *Sesarma sp.* (Table I). Of the 11 bands in *Sesarma sp.*, 6 were variable, and the 5 qualitatively invariant bands still varied in density (Fig. 3). Although only 4 of the 9 bands were qualitatively variable in *S. reticulatum* (Fig. 2), all but one of the invariant bands varied in density (Fig. 3).

With the exception of the infrequent band 14, the variation in *S. reticulatum* was limited to bands 3, 4, and 5, which varied concomitantly in the classic pattern expected of the products of alleles encoded at the same lo-

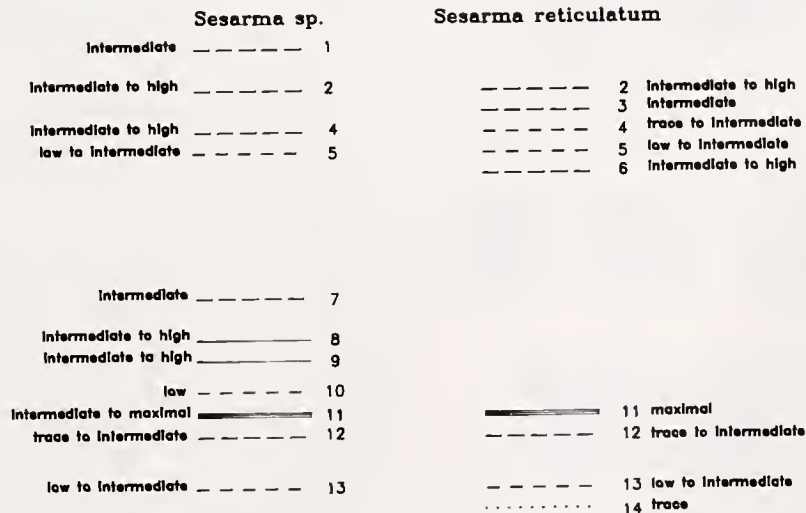


Figure 3. Diagrammatic representation of the electrophoretic bands in *Sesarma*. If no range is specified, then band density did not vary.

Table 1

Phenotypes observed in *Sesarma*

A. <i>Sesarma reticulatum</i>																									
2	4	6				11	12	13		(70)		2	4	5	6			11	12	13	(3)				
2	3	4	6						11	12	13				5	6		11	12	13	(1)				
2		4	6						11	12	13	14				6		11	12	13	(19)				
2	3		5	6					11	12	13														
B. <i>Sesarma</i> sp.																									
2	4				8	9		11		13		(18)		1	2	4	5		8	9	11	12	13	(5)	
2	4				8	9		11	12	13		(41)			2	4	5		8	9	10	11	12	13	(3)
2	4	5			8	9		11	12	13		(5)			2	4			8	9	10	11	12	13	(5)
2	4	5			9			11	12	13		(3)		1	2	4	5		9			11	12	13	(2)
2	4			7	8	9		11	12	13		(2)			2	4			9			11	12	13	(15)
2	4				9	10		11	12	13		(2)			2	4	5		8	9	10	11		13	(3)
1	2	4			8	9		11	12	13		(2)													

The parenthetical number is the frequency (%).

cus. Each of these three bands was expressed either alone or together with one of the other two, but all three were never found together (Table 1); nor were all three ever absent coincidentally. In general, density was highest when one of the three occurred alone (Fig. 4). Of the

members of the triplet, band 4 was by far the most frequent (Fig. 2). In *Sesarma* sp., band 4 was invariant, and its density did not clearly decrease when band 5 was expressed. The third member of the *S. reticulatum* triplet (band 3) was never found in *Sesarma* sp.

In *Sesarma* sp., bands 8 and 12 also appeared to vary as expected of the products of a single locus. Again, each was found alone or together with the other, but one or both were always expressed. In this case, the variation in density (Fig. 3) appeared to be independent. The frequencies of each were quite similar in *Sesarma* sp., but band 8 was never found in *S. reticulatum* (Fig. 2).

Because this is the only of now several investigations in which we have encountered allozyme-like variation, we have chosen not to estimate "gene" frequencies and Hardy-Weinberg ratios until the genetic character of the observed variation can be corroborated. We do, however, report the frequencies of each phenotype in the *Sesarma* complex (Table 1).

Uca minax

No difference was found between the Atlantic cold temperate zone animals (*n* = 161) collected at the several sites in Virginia; therefore, they are treated here as a single sample. Similarly, no difference was found between the 22 Gulf coast animals collected in Louisiana and the 49 collected in Mississippi, which are also treated here as a single sample. In each area, that is, Gulf coast and Atlantic cold (Virginia) temperate zone, a total of 17 bands were found. In any one sample, each band co-migrated to the same position as did a counterpart in the other two samples (Fig. 5). In other words, no marker bands were diagnostic of a particular sample. In addition, the modal number of bands expressed in each sample is the same (Fig. 6). Nonetheless, each sample differs significantly from the other two.

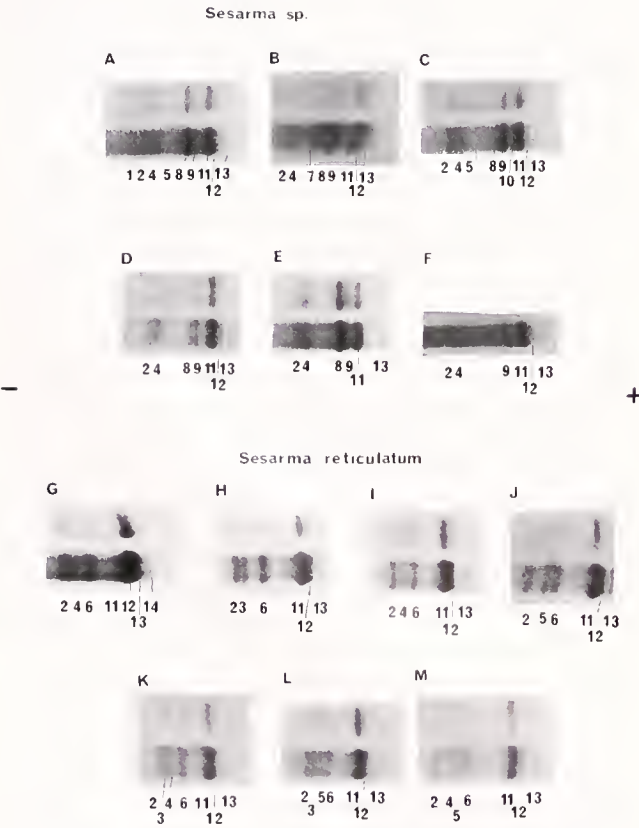


Figure 4. Examples of the variation in *Sesarma*. With the exception of F, each panel shows higher (bottom) and lower (top) concentrations of the material from the same individual.

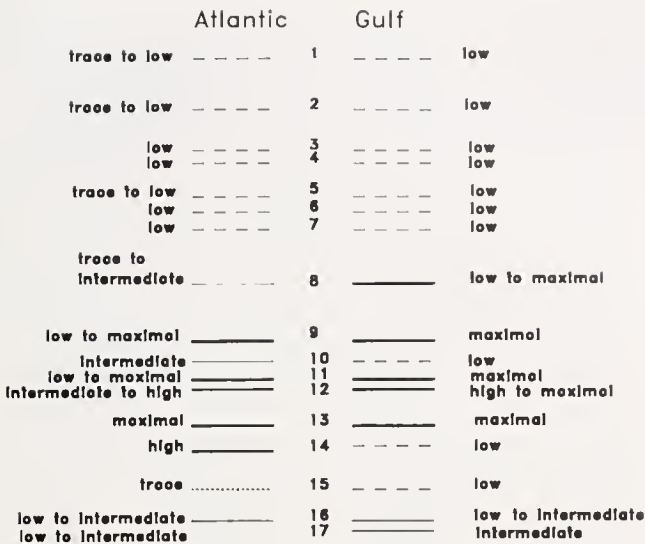


Figure 5. Diagrammatic representation of the electrophoretic bands in *Uca minax*. If no range is specified, then band density did not vary.

First, the frequencies of the bands differ (Fig. 7). Pairwise comparisons were made in a contingency test of independence. Because some cells in the Atlantic warm temperate zone sample were empty, the Chi-square statistic was used. Each of the two Atlantic samples differs from the Gulf sample at a probability level several orders of magnitude less than $P = 0.001$. Second, the variation in band density was similar in the two Atlantic samples, but it differed from that in the Gulf sample (Fig. 5). Perhaps most conspicuous, the phenotypes of the major bands, defined as those that comprise the majority of the material present, qualitatively distinguished the two Atlantic samples from the Gulf sample (Table II). Although the phenotypes of the major bands in the two Atlantic samples were the same, their frequencies differ significantly. Again, in a pairwise comparison by a contingency test of independence, the two differ at about $P = 0.001$.

Each of the three samples exhibited considerable poly-

morphism, with a very large number of phenotypes (Table III, Fig. 8). The sample from the Gulf coast, the smallest of the three ($n = 71$), had the smallest number of phenotypes (26). The sample from the warm temperate zone on the Atlantic coast, only slightly larger ($n = 73$), had a similar number of phenotypes (28). The sample from the cold temperate latitudes on the Atlantic coast, which was the largest ($n = 161$), exhibited a proportionately larger number of phenotypes (56), which is somewhat surprising. One would expect the rate of increase to diminish with increasing sample size.

In all three samples, the bands that occurred in either high densities or high frequencies were clearly positive for Cu. Bands 1, 2, 6, and 7 were neither clearly positive nor clearly negative because the combination of low density and low frequency precluded repeatedly overloading the gels for the less sensitive Cu detection procedure.

The only band that was invariant in all three samples was 14 (Fig. 8). However, bands 3, 4, and 13 were absent in only a very few individuals, and the variation in band 16 was confined to the Gulf sample (Fig. 7, Table III).

None of the qualitative variation of any one band was coupled to that of any other in a way that clearly suggests encoding at a common locus (Table III). One instance of coupled quantitative variation was noted: in the two Atlantic samples, the densities of bands 9 and 11 always varied concomitantly and directly.

Discussion

On the basis of allozymic variation at 13 polymorphic loci, Felder and Staton (1994) concluded that the genetic distance between Atlantic and Gulf of Mexico samples of *Sesarma reticulatum* (Say) is similar to that found in recently speciated populations of other brachyurans. The data strongly supported the authors' earlier conclusions, which were based on coloration, osmotic responses, and reproductive chronology (Zimmerman and Felder, 1991; Staton and Felder, 1992). All allozyme differences between samples of the two disjunct popula-

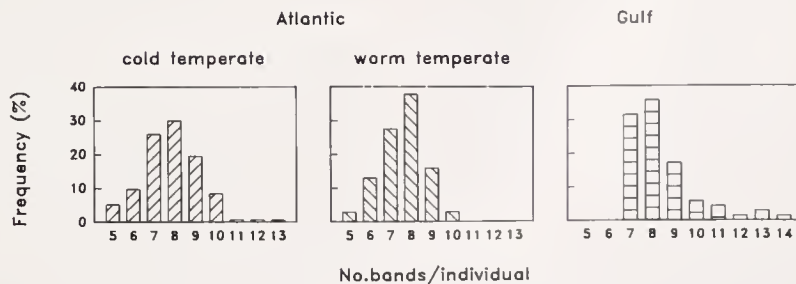


Figure 6. The frequency distribution of the total number of bands found in an individual of *Uca minax*. Values of n are 161 for the cold temperate zone sample, 73 for the warm temperate sample, and 71 for the Gulf sample.

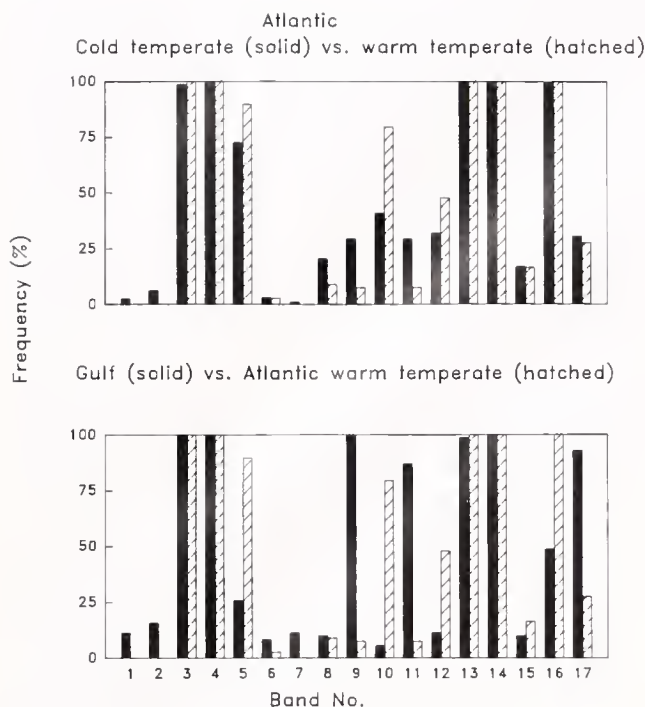


Figure 7. The frequencies of each band in *Uca minax*. Sample sizes as in Fig. 5.

tions, which they designated *S. reticulatum* and *Sesarma* sp., were quantitative, although several morphs completely absent from one coast existed in very high frequencies on the other.

The Hcs of the two *Sesarma* samples investigated here differ qualitatively. Band 9, found in all individuals of *Sesarma* sp., was totally absent from the *S. reticulatum* sample. Therefore, the present findings not only support the hypothesis of speciation, they also suggest that the Hcs differ more than the 13 polymorphic enzymes (much less an additional 7 enzymes, which were monomorphic in both species). This suggestion is constrained, however, by the use of different sampling sites on the Atlantic coast in the two investigations. Whereas Felder and Staton (1994) examined only warm temperate zone members of *S. reticulatum*, we examined only cold temperate zone representatives. Populations in the two biogeographic provinces on the Atlantic coast are by no means disjunct, but Cape Hatteras, which separates them, is a major faunal discontinuity (e.g., Vernberg and Vernberg, 1972) and, in several taxa, populations found on either side of it differ (e.g., *Uca minax* in the present investigation).

Felder and Staton (1994) reached different conclusions for *Uca minax*. In this case the allozyme variation was deemed insufficient to warrant specific separation. Although the inference was also based on 13 polymorphic loci, the variation at 9 of them was quite small. The

only pertinent morphological evidence in this species is the intensity of the red coloration on the articles of the chelipeds and legs, which is strongly developed in Atlantic and western Gulf populations but, oddly, not in eastern Gulf populations. No comparative information on physiological or reproductive responses is available. As indicated above, our samples of eastern and western Gulf members of *U. minax* did not differ, indicating no relationship to the color patterns. Most of our sample originated from a Mississippi site where the color difference from Atlantic animals is maximal, and the remainder was collected in Louisiana, where the pattern more closely resembles that found in Atlantic members of the species. A similar conclusion was reached by Felder and Staton (1994), whose dendrogram based on allozymic differences does not reflect the color patterns.

Regardless, we still found clear, though primarily quantitative, differences in the overall banding patterns of the He monomers in each of the three samples analyzed. The same number of Cu-containing bands was found in Atlantic and Gulf animals, and each band in one sample co-migrated with a counterpart in the other. The most probable inference is that the co-migrants represent the same polypeptides. Nonetheless, the frequencies of the bands are very different in each sample. The overall differences between the two Atlantic samples are no greater than those observed within *Callinectes sapidus*, which are not genetically fixed in the adult stage (Mangum, 1990; deFur *et al.*, 1990). In contrast, the differences between either Atlantic sample and the Gulf sample are clearly greater than those in *C. sapidus*, which can be induced by environmental change. Although cross-acclimation experiments should be performed in

Table II

Frequencies of the phenotypes of the major bands, defined as those that comprised the majority of the total amount of material, in *Uca minax*.

				Atlantic		Gulf
				cold temperate	warm temperate	
(% total)						
12	13	14		19.9	41.7	0
8	10	13	14	1.2	1.4	0
9	11	13	14	23.0	7.0	0
13	14			55.9	50.0	0
11	13			0	0	1.4
9	10	13		0	0	2.9
9	11	13		0	0	84.3
9	12	13		0	0	4.3
10	12	13		0	0	1.4
10	12	14		0	0	1.4
8	9	11	13	0	0	1.4
9	11	12	13	0	0	2.9

Table III

Phenotypes observed in Uca minax

A. Atlantic										2. Warm temperate									
1. Cold temperate										3 4									
3 4										13 14 16									
3 4 5										12 13 14 16									
3 4										13 14 16									
3 4 6										12 13 14 16 17									
3 4										13 14 16 17									
3 4 10										13 14 16									
3 4 9 11										13 14 16									
3 4 5 10										13 14 16 15 17									
3 4 5										12 13 14 16 17									
3 4 10										12 13 14 16 17									
3 4 5 6 10										13 14 15 16 17									
3 4										12 13 14 16 17									
3 4 5										13 14 16 17									
3 4 5										13 14 15 16									
3 4 5										12 13 14 16 17									
3 4 5										12 13 14 16 17									
3 4 5 9 11										13 14 16									
3 4 5 10										13 14 16 17									
3 4 5 10										12 13 14 16									
3 4 5 9 11										13 14 15 16									
3 4 5 9 11										13 14 16 17									
3 4 5 8 9 11										12 13 14 16									
2 3 4 5										13 14 16 17									
3 4 5										13 14 15 16 17									
3 4 5 8 10										13 14 16									
3 4 9 11 12										13 14 16									
2 3 4 9 11										13 14 16									
3 4 5 8										13 14 15 16									
3 4 5 9										12 13 14 16 17									
3 4 5 8										13 14 16 17									
3 4 5 9										13 14 15 16									
3 4 5 10										12 13 14 16 17									
3 4 5 8 9 11										13 14 16									
3 4 6 9 11										13 14 16 17									
3 4 8 10										12 13 14 15 16 17									
3 4 5 6 9 11										13 14 16									
3 4 5 8 10										13 14 15 16									
3 4 5 8 10										13 14 16 17									
3 4 5 8 9 11										13 14 16 17									
3 4 5 8 10										12 13 14 16									
3 4 5 8 9 11										13 14 15 16 17									
2 3 4 5 8 9 11										13 14 16									
1 2 3 4 5 6 7 8 10										12 13 14 16									
3 4 5 9 11										13 14 15 16 17									
3 4 5 8 10										12 13 14 16 17									
3 4 5 8 9 11										12 13 14 16									
1 3 4 5 8 10										13 14 15 16 17									
3 4 5 10										12 13 14 15 16 17									
3 4 5 8 9 11										13 14 16 17									
3 4 5 8 10										13 14 15 16 17									
3 4 5 8 10										12 13 14 15 16									
3 4 5 7 8 10										13 14 16 17									
3 4 5 8 10										12 13 14 15 16 17									
1 2 3 4 5 8 9 11										13 14 15 16									

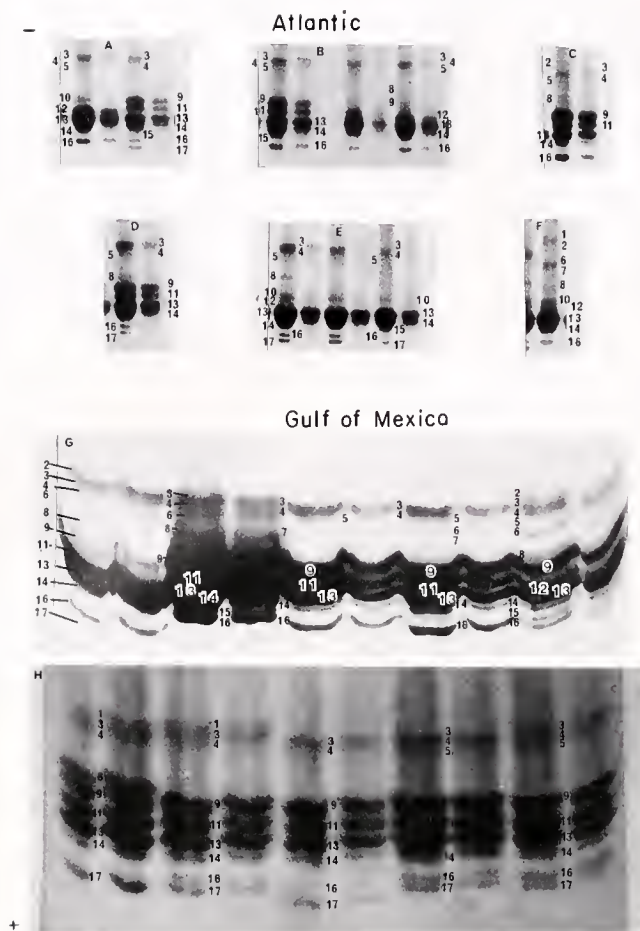


Figure 8. Examples of the variation in *Uca minax*. Top: Atlantic animals. With the exception of panel F, the lanes are arranged in pairs. Each pair of lanes, from left to right, shows a higher and a lower concentration of material from the same individual. Bottom: Gulf of Mexico animals. All lanes are arranged in pairs showing two concentrations of material from the same individual. With the exception of the pair at the far left, which shows first the lower and then the higher concentration, each pair alternates first the higher and then the lower concentration, as above.

U. minax, we suggest tentatively that the differences may reflect, at least in part, genetic divergence of the disjunct Atlantic and Gulf populations. This suggestion, if true, also supports the hypothesis that the Hcs diverge early in speciation.

We note that the differing magnitudes of the divergence in the two complexes examined here agree with the findings of Avise and co-workers as well as with those of Felder and students. On the basis of results for 19 species ranging from bivalves to chelicerates to teleosts, Avise (1992) found no evidence of a uniform molecular clock that measures the rate of divergence of Gulf and Atlantic populations.

We also suggest tentatively that very early divergence of the Hcs may be accelerated when the products of speciation must adapt to different thermal regimes (see Reese and Mangum, 1994), such as those found in the

warm temperate (Gulf) and cold temperate (Atlantic, north of Cape Hatteras) zones.

Perhaps most important from a physiological point of view, the Atlantic and Gulf samples of *U. minax* are qualitatively differentiated by the phenotypes of the major bands, those that are most likely to influence respiratory properties. In addition, this inference remains valid when the comparison is restricted to Atlantic warm temperate zone vs. Gulf samples. The thermal regimes experienced by these two populations differ relatively little, certainly far less than the thermal regime experienced by either population and that experienced by Atlantic cold temperate zone populations.

Deevey (1950) suggested that environmental temperature is the primary factor responsible for the disjunction of Atlantic and Gulf species, a widespread phenomenon in a number of animal phyla (see also Avise, 1992). In

his scenario, disjunct populations are relicts of the retreat of the last glacier. The concomitant rise in temperature on both coasts around the Florida peninsula caused the retreat northwards of temperate zone species and their replacement in Florida by tropical and subtropical species invading from the south. Barnwell and Thurman (1984) correctly pointed out that biological factors such as the attendant changes in vegetation must also play a role in the disjunction of fiddler crab species. Whereas *U. minax*, for example, inhabits *Spartina* salt marshes, the species of *Uca* now found in subtropical regions of the Florida peninsula are mangrove dwellers.

Although we do not doubt the importance of habitat, we believe that temperature is likely to be extremely important in the present case. Temperature is the factor most often associated with divergence of respiratory properties among the Hcs of closely related species (Reese and Mangum, 1994). Consequently, the thermal sensitivities of the respiratory properties of Hcs expressing the most frequent phenotypes of major bands in *Uca minax* and the two *Sesarma* species will be of considerable interest.

Acknowledgments

Supported by NSF DCB 88-16172 (Physiological Processes). Some of the material was collected while the first author held a visiting appointment at the University of Southwestern Louisiana. We thank Lewis Deaton, Richard Heard, and Harriett Perry for their prodigious efforts and invaluable help in collecting animals in the bayous, which far exceeded normal courtesy. We also thank D. L. Felder for collecting the *Sesarma* sp. material from Joseph's Harbor, and K. A. Callicott for collecting the *Uca minax* material from North Carolina. CPM was ably assisted by T. L. Atkins in the PAGE of material from *Uca minax*.

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Particle Capture by the Gills of *Dreissena polymorpha*: Structure and Function of Latero-frontal Cirri

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Abstract. Microscopic techniques were used to examine the role of gill cirri in particle capture by *Dreissena polymorpha*. The latero-frontal cirri, formed from two fused ciliary plates, consist of about 40 pairs of cilia. Each cilium in the plate contains a typical 9 + 2 axoneme in the fused region of the cirrus, but the structure of the axoneme in the long, free ciliary tips is reduced. The cilia in a cirrus are graded in length, with the shortest cilia positioned frontally. The cirral cilia move in unison, allowing the cirrus to move from a flexed position with its tip arched over the front of the gill filament, to an extended position with the cirrus projected in the plane of the latero-frontal cell and extending across the interfilament space. In the latter position, the free ciliary tips of opposing and neighboring cirri form a "trap" (net) with a spacing of about 0.5 μm . Observations with laser confocal microscopy indicated that these structures can physically trap particles $\leq 1 \mu\text{m}$ in diameter. Particles captured by the extended cirri are moved to the frontal surface of the gill, where the cirri are swept by the lateral-most frontal cilia. During cirral movement the shift from extended to flexed position is, in part, achieved by the base of the cirrus pivoting at a hinge region. Morphologically, the hinge region shows axonemal specializations that consist of electron-dense plates and other structures of undefined function that may be important in the overall movement of the cirrus. In addition to trapping by cirri, we also observed particles moving in the water currents, particularly in the frontal current located over the apical surface of the filament, suggesting that some particles are captured in these water currents without being physically trapped. Probably, therefore, trapping by the

cirri and establishment of water currents by the filaments both participate in the interception of particles by *Dreissena polymorpha*.

Introduction

Understanding of suspension feeding in lamellibranch bivalves requires a knowledge of the factors responsible for water movement through the mantle cavity and across the gills. In addition to water flow characteristics, the mechanisms of particle capture, transport, selection, and ingestion in a particular species define the abilities and the limitations of suspension feeding in that species. The organ primarily responsible for particle capture is the gill. Morton (1983) reviewed this topic, and describes the filtration (suspension feeding) mechanism to be "largely a function of eulaterofrontal cilia, although mucus and patterns of water flow over the surface of the ctenidia may play an additional role in filter feeding." The generalized role of water flow patterns in suspension feeding has been reviewed by Jørgensen (1990), and the role played by mucus in particle manipulation has been elucidated by Beninger and colleagues for various marine species (Beninger *et al.*, 1991, 1992, 1993; Ward *et al.*, 1993), and by Tankersley and Dimock (1993) for a freshwater unionid.

In eulamellibranch gills, particles are captured from water that is driven across the gill filaments by the motion of the lateral cilia on each side of the filaments (Gibbons, 1961; Jørgensen, 1982, 1989). Latero-frontal cells and their complex motile organelles, either cilia or cirri composed of many fused cilia, are located between the frontal surface of the filament and the lateral ciliated cells. Atkins (1938) describes the cirri in a variety of bivalves and notes their widespread occurrence among lamellibranchs. Variation in the latero-frontal ciliary appendages of different bivalves ranges from single cilia to

Received 5 January 1995; accepted 17 April 1996.

Abbreviations: CC—cirral cilia; PW—pondwater; SEM—scanning electron microscopy; TEM—transmission electron microscopy.

those formed by two fused ciliary plates containing as many as 40 cilia per plate (Atkins, 1938). The cilia forming the fused cirral plates have free ciliary tips that form what appear to be nets or sieves in the interfilament space between gill filaments (Atkins, 1937; Moore, 1971; Owen and McCrae, 1976; Ribelin and Collier, 1977).

Cirral ultrastructure and the structure of the interfilament sieve has been described for many marine species (Dral, 1967; Gibbons, 1961; Moore, 1971; Owen, 1974; Owen and McCrae, 1976; Ribelin and Collier, 1977), and the beat pattern of an individual cirrus on the gill of *Mytilus edulis* has been described by Dral (1966, 1967). Several attempts to correlate the efficiency of particle capture with species differences in cirral structure have led to conflicting interpretations and conclusions.

If one assumes that 1 μm particles should be retained at 100% efficiency by cirral nets, then the experimental results of some feeding experiments do not support a cirral particle capture mechanism (Vahl, 1973; Møhlenberg and Riisgård, 1978; Riisgård, 1988). Others have concluded that the latero-frontal cirri participate in mechanical filtration (Atkins, 1938; Dral, 1967; Moore, 1971; Owen and McCrae, 1976; Silvester and Sleight, 1984). But the lack of cirri in some bivalve families, as well as hydrodynamic considerations, led Nielsen *et al.* (1993) to suggest that cirri may be specialized structures that improve the retention efficiency of the gill for small particles. Small particle retention efficiency varies among species with cirri of different sizes (Vahl, 1973; Møhlenberg and Riisgård, 1978; Kryger and Riisgård, 1988; Silverman *et al.*, 1995), with those species having complex cirri showing the highest efficiency. To date, the functions of cirri in particle capture have been attributed to their role in complex current formation, their contribution to overall gill hydrodynamics, or to mechanical filtration. These roles are not mutually exclusive, and perhaps a combination of mechanisms allows bivalves with complex cirri to retain smaller particles.

Dreissena polymorpha is a eulamellibranch filter feeder capable of removing particulates <1 μm in diameter (Sprung and Rose, 1988). Particles filtered include single-celled algae, diatoms, and some bacteria (Mikheev and Sorokin, 1966; Morton, 1971; Ten Winkel and Davids, 1982; Jørgensen *et al.*, 1984; Silverman *et al.*, 1995). In this paper, we use laser confocal microscopy to describe cirri and their movements in *D. polymorpha*. The gill cirri of *D. polymorpha* are complex as described by Atkins (1938). An advantage of laser confocal microscopy is its selectable image plane and depth of field coupled with a high resolution (approaching 0.2 μm). The results indicate that, in isolated gill preparations, cirri participate in the capture of small particles.

Materials and Methods

Preparation and fixation of gill tissue for microscopy

Dreissena polymorpha were collected from Lake Erie and from the Mississippi River near Baton Rouge, Louisiana. Animals were maintained in the laboratory at 4–15°C in pondwater and were acclimated to 23°C for 3–5 days before use (Dietz *et al.*, 1994). During this study, the gills from more than fifty animals were examined microscopically.

The standard tissue fixation was accomplished by inserting a syringe needle into the byssal opening in the shell, injecting phosphate-buffered 2% glutaraldehyde (pH 7.2) into the intact animal, and then immersing the entire animal in fixative for 1 h. The concentration of the phosphate buffer was adjusted to match the measured osmolality of *D. polymorpha* blood, about 35–45 mosm. The gills of all freshwater bivalves are extremely sensitive to the osmolality of the fixative. For *D. polymorpha*, an osmotic imbalance of 10 mosm in either direction of isosmolarity resulted in an unsuitable preparation (see Silverman *et al.*, 1996). Following the 1-h immersion in fixative, the animal was dissected and 1–6 mm gill strips were placed in fresh, buffered glutaraldehyde for an additional hour. Alternatively, some gills were carefully dissected without pre-fixation and pinned in a desired orientation before a 1-h glutaraldehyde fixation. All tissues were rinsed in isosmotic phosphate buffer and post-fixed for 1 h in 1% osmium tetroxide. Tissues for light and transmission electron microscopy (TEM) were dehydrated in a graded alcohol series and embedded in LR White (London Resin Co., Reading, England) methacrylate resin cured at 60°C (Gardiner *et al.*, 1991a).

Gills were sectioned with a Reichert-Jung Ultracut E ultramicrotome at a thickness of 0.5–2.0 μm for light microscopy and at 60–90 nm for electron microscopy. Sections for light microscopy were stained with borate buffered 1% toluidine blue containing 0.5% borax and then examined with a Nikon Microphot-FXA microscope equipped with Planapochromat lenses. Sections for TEM were stained with 2% uranyl acetate for 10 min followed by lead citrate for 1 min (Reynolds, 1963). Thin sections were examined with a JEOL 100CX transmission electron microscope operating at 80 kV.

For observation with the scanning electron microscope (SEM), some gills were pre-treated with 10^{-6} M serotonin. As in unionid gills (Gardiner *et al.*, 1991a), 10^{-6} M serotonin increases cirral beat and relaxes the branchial musculature in *D. polymorpha*, thus increasing ostial diameter and interfilament distance (Gardiner *et al.*, 1991a, b). This effect facilitated the observation of the motile organelles on the filament surface. For SEM, fixation was as described above with all specimens dehydrated in a graded alcohol series. Specimens were

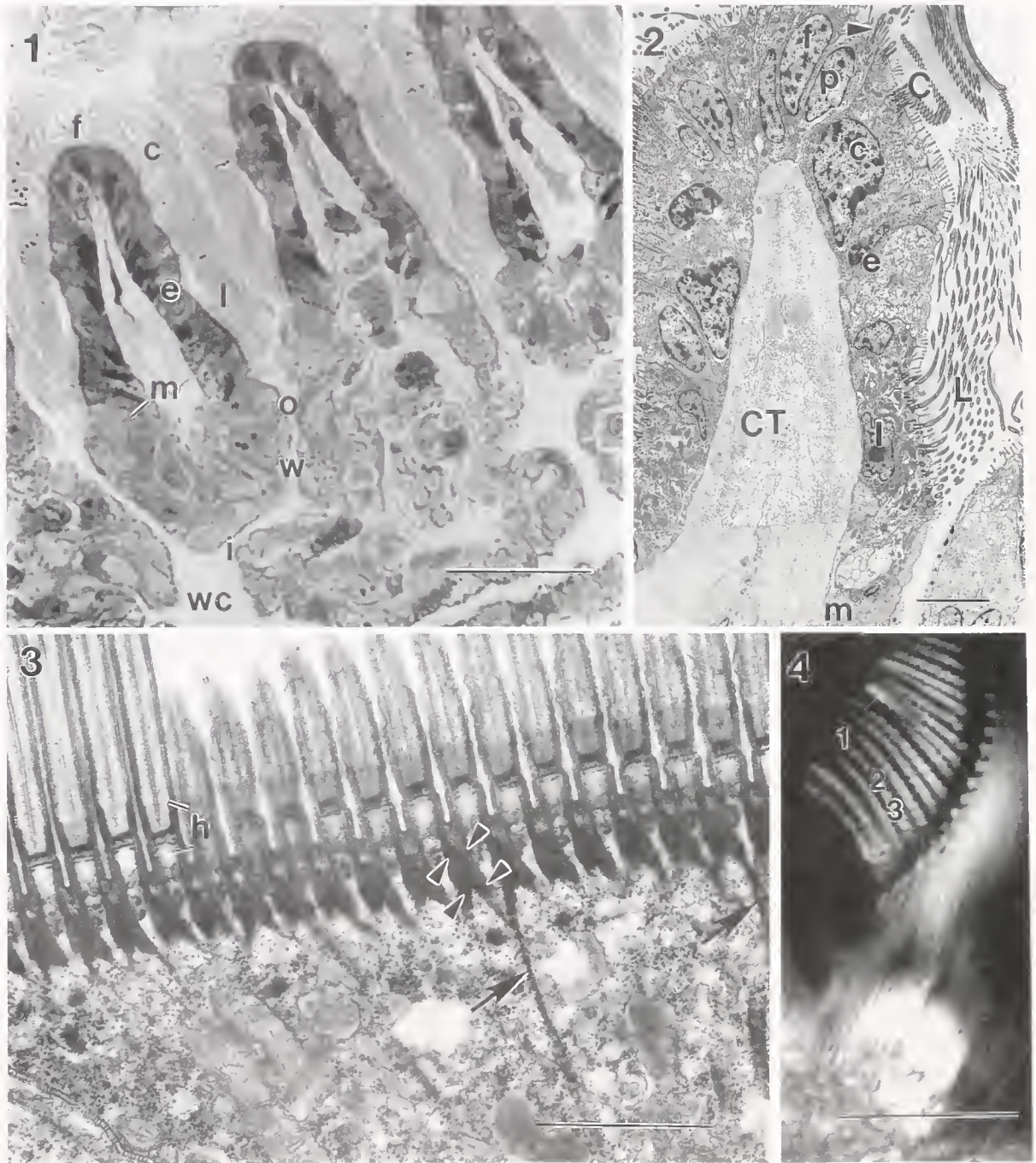


Figure 1. A section through the gill of *Dreissena polymorpha* showing portions of three filaments stained with toluidine blue. Note the relative position of frontal cilia (f), latero-frontal cirri (c), and lateral cilia (l), along the filament. In addition to the three cell types with cilia, there is a set of indifferent epithelial cells (e) between the lateral ciliated cells and the latero-frontal cells. Water moves from the surface of the filaments through the ostial opening (o) into the water canal (w), through the internal ostia (i) and into the central water channel (wc). The water canals on both sides of a filament enter the central water channel through one common internal opening. Note that there are no goblet-type mucus (m)-producing cells among the cells making up the surface epithelium of the filament, but they are present at the filament base near the ostial opening and in the central water channel. Bar = 25 μ m.

Figure 2. An electron micrograph collage showing the epithelial cells of the filament: frontal epithelial cells (f); pro-lateral-frontal epithelial cells (p), latero-frontal cirral cells (c); indifferent epithelial cell (e); lateral ciliated cell (l); mucus cell (m), cirri (C), pro-latero-frontal cilia (arrowhead), and lateral cilia (L). The connective tissue skeleton of the filament (CT) is also notable. Bar = 5 μ m.

wrapped in lens paper to prevent curling, critical point dried (Denton Vacuum, Inc), and mounted on stubs with double-sided carbon tape. Specimens were sputter-coated with gold and palladium (20 nm) and viewed with a Cambridge 2000 scanning electron microscope operating at 10–15 kV.

Gill visualization by laser confocal microscopy

Because gill tissue displays some autofluorescence and reflectance, observations of the gill by laser confocal microscopy can be made either with or without fluorescent markers. For specific enhancement of cilia and cirri, FITC-colchicine (FITC-C; Molecular Probes, Inc.) was used. Gills were carefully removed from an animal and placed in freshwater clam Ringer's solution (Gardiner *et al.*, 1991b). Some of these gills, unfixed, were split along the septa into two lamellae by microdissection. The cilia and musculature of gills prepared in this way remained active for more than 12 h. Gill strips to be fluorescently labelled were separated and placed in 20 $\mu\text{g}/\text{ml}$ FITC-C for 10–30 min. During exposure to fluorescent dyes, the gills were protected from light. The gill strip was removed from the marker dye, washed in several changes of Ringer's solution, and maintained in the dark until used.

Gills were oriented and mounted on a slide under a dissecting microscope. Some gills were mounted on open mesh Nitex screen (125 μm mesh), which allowed water to flow more readily across the gill. A coverslip supported at the edges by silicone vacuum grease was suspended over the preparation. This arrangement was necessary to keep the coverslip from interfering with ciliary and cirral motion. The specimen was examined with a Noran Instruments, Inc., laser confocal system mounted on a Nikon Optiphot microscope equipped with fluorescence objectives. The excitation wavelength was 529 nm from an argon ion laser for all preparations, and an FITC barrier filter was used. The video image consisted of both a reflective and a fluorescent component. Images were captured and analyzed with Odyssey InterVision (Noran Instruments, Inc.) hardware and software on a Silicon Graphics Indy computer.

In other experiments, fluorescent beads (FITC) of 1.0 μm and 0.75 μm diameter were used to track water flow and particle handling by the gill. The FITC-beads were added to the slide at the dorsal end of the gill. This allowed particle movement to be tracked across the gill, and particle interaction with the surface components of the gill to be visualized. In these experiments, no FITC-colchicine was used, and we relied on autofluorescence and reflectance to visualize the cirri.

Measurement of ciliary beat frequency

Beat frequencies of the cirri and lateral cilia were determined with an Intervision software program that records changes in brightness over time. With the confocal microscope focused at the appropriate ciliary group, a small function box was superimposed on the image such that a single cirrus, cilium, or a group of lateral cilia passed through the box with each beat. Because cilia are imaged as bright structures on a darker background, a graph of continuous beating could be captured over any time interval and displayed as peaks in the digital "strip chart" plotted against time. Over a given period, the peaks were counted for three areas in five excised non-stimulated gills and the values averaged to give the beat frequency for the cirri and lateral cilia. Because of variability between and even within gills, we have reported these values as range estimates rather than as statistical averages. The isolated gill preparations used here are differentially sensitive to neurohormonal agents and their pharmacologic analogs (unpubl. obs.; Gardiner *et al.*, 1991a, b). We have not reported the effects of neurohormonal agents in this study. We frequently used serotonin to stimulate quiescent cilia to verify tissue viability and responsiveness.

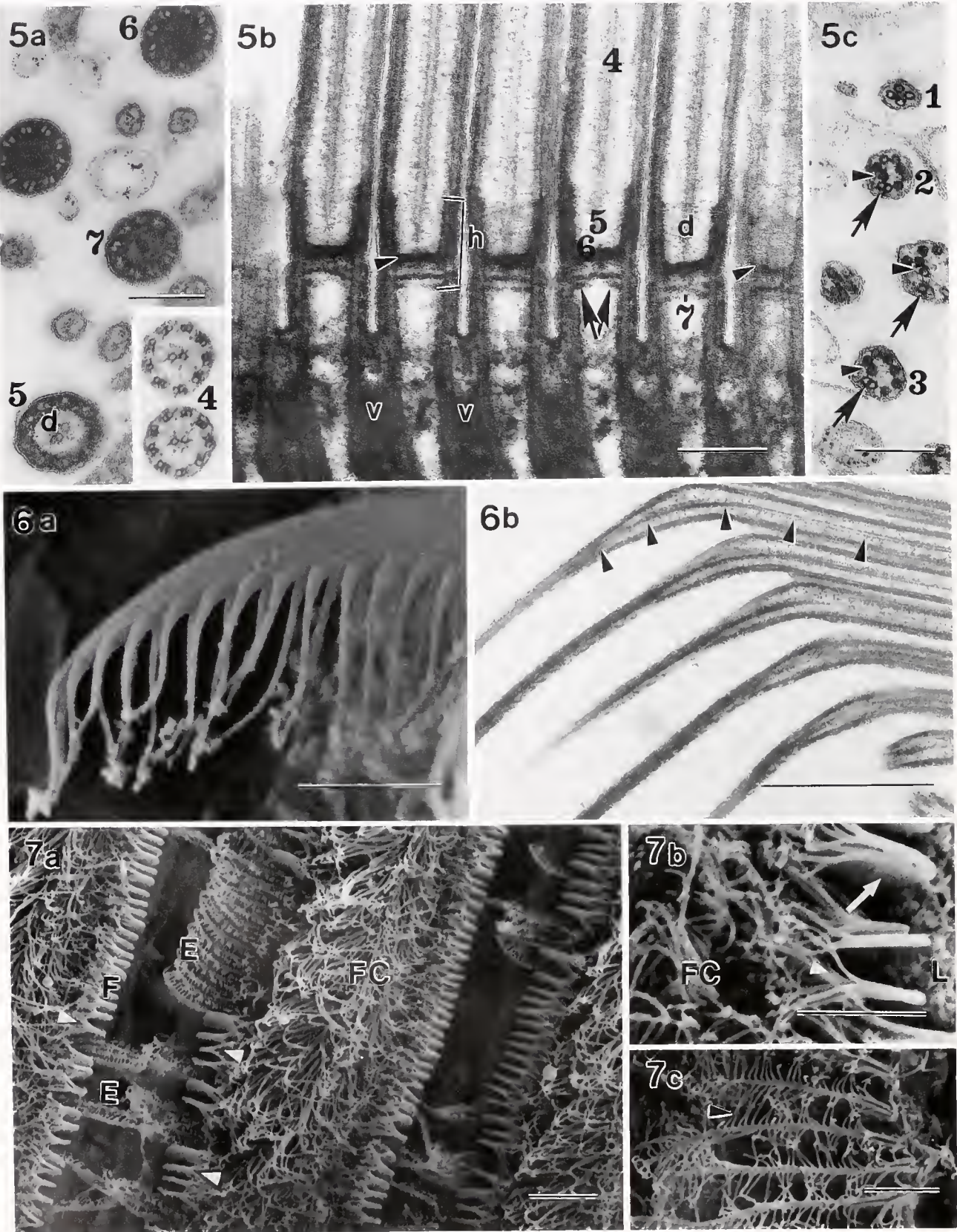
Results

General structure of the gill filament of D. polymorpha

Morton (1993) has described the structure of the *D. polymorpha* gill as conforming to the general eulamelli-branch pattern (Fig. 1). Most descriptions of water flow

Figure 3. Transmission electron micrograph of the apical portion of a latero-frontal cell showing the basal portion of a single cirrus composed of numerous cilia. The extensive system of rootlets (arrows) underlying the cirrus is evident. Note that these rootlets are anchored in electron-dense structures that envelop the microtubules at the base of each cilium (arrowheads). These dense structures link the microtubules on the right side of one cilium to the left side of the next cilium. The hinge or neck region (h) of each cilium is also visible. Bar = 1 μm .

Figure 4. Single Z-plane image of a cirrus, one of a series of images captured every 33 ms by laser confocal microscopy. This cirrus was moving toward the flexed position where it would partially cover the frontal cilia. The fused plate component of the cirrus consisting of individual cilia is located at the bottom of the micrograph. The free tips of the individual cilia are at the top of the micrograph. Positions 1, 2, and 3 on the free tips show reduced microtubule numbers from the normal "9 + 2" axoneme structure and correspond to 1, 2, and 3 in Figure 5c. Bar = 5 μm .



across such gills indicate that the lateral ciliated cells create the force for water flow through the interfilament ostia (Gibbons, 1961; Jørgensen, 1981, 1982, 1989). A detailed examination of the filament epithelium of *D. polymorpha* revealed that all cells contain microvilli, and that there are at least five different cell types (Figs. 1, 2). Three of these cell types have motile appendages: the frontal and the lateral ciliated cells contain cilia as individual structures, but the latero-frontal cells contain cirri composed of fused cilia. The frontal cells closest to the latero-frontal cells have longer cilia than the rest of the frontal cells, and these correspond to the pro-lateral-frontal cells described in many species with large latero-frontal cirri (Atkins, 1938). There were 3–4 lateral ciliated cells and a latero-frontal cell in a cross section through the filament (Fig. 1). A slightly oblique cross section may show multiple latero-frontal cells. Between the lateral ciliated

cells and the latero-frontal cells is a nonciliated cell (Fig. 2). Between the lateral ciliated cells and the base of a filament (near the ostial opening) are other nonciliated cells (Fig. 1). The only goblet-shaped mucus cells seen were located at the base of the filament surrounding the external ostia leading to the central water channel of the gill (Figs. 1, 2). The water channel epithelium also contains many mucus cells, especially around the internal ostia and on the septa (not shown).

Laser confocal microscopy allowed observation of the characteristic beating patterns associated with each of the three ciliary appendage types. The frontal cilia on the outer surface of the filament propel material ventrally toward the food groove on the gill margin. Each frontal cilium displays a typical ciliary beat consisting of a power stroke and recovery stroke (Sleigh, 1962). Observation of the lateral ciliated cells confirmed a powerful rhythmic,

Figure 5. High magnification transmission electron micrographs through various regions of a cirrus. Cross sections through various regions of the cilium are identified by number (1–7) increasing from tip to base. (a) Cross-sectional images through cirral components; individual cilia from a cirrus have separated during fixation, but cross-sectional images at different levels allow a comparison with the longitudinal images. The inset shows the normal 9 + 2 axonemal pattern seen throughout the fused portion of the cirrus. (b) A high magnification longitudinal micrograph through the hinge region of Figure 3. (c) Cross section of a cirrus made in the region of the ciliary tips. Note in the longitudinal section that the hinge region [h] is narrower than the rest of the axoneme (Figs. 3, 5b). The normal axoneme and its corresponding cross section (in Fig. 5a, b) are indicated by 4. The hinge region is 0.2 μm long; for most of this length, the axonemal microtubules are surrounded by an electron-dense material (5 in Fig. 5a, b). The central microtubular doublets are present in this region (d). Toward the base of the cilia, the hinge region appears to be continuous with an electron-dense plate (arrowhead) such that the electron-dense material actually forms a cup on the basal end of the cilium proper. The outer doublets, but not the central pair of microtubules, appear to penetrate this plate (6 in Figs. 5a, b). Below this plate is a second thinner electron-dense plate (7 in Figs. 5a, b). Finally, a cone of additional electron-dense material (arrows) connects the outer microtubular doublets to the center of the thick electron-dense plate immediately below the level where the central doublets end. The connections of outer microtubular doublets at the base of the cilia described in Figure 3 are also visible (v) connecting adjacent cilia. The larger cross sections in 5c contain both microtubular doublets and singlets. These ciliary cross sections are distal to the shoulder region and the number of singlets is less than the original nine microtubular doublets of the axoneme. Note the eccentric position of the central doublet (arrowhead) and its position with reference to the remaining outer doublet (arrow). Progressing toward the ciliary tip the diameter and number of microtubules in the axoneme decreases. The relative positions at which these axonemal structures are present is identified as 1, 2, and 3 in Figure 4. Bars = 0.2 μm .

Figure 6. Electron micrographs of the shoulder region of a cirrus where the free tips of the cilia emerge from the fused body region. (a) The scanning electron micrograph shows the abrupt angle formed between the free ciliary tips and the fused body of the cirrus. (b) A comparable transmission electron micrograph of this same region indicates that, at the shoulder region, the central doublet (arrowheads) does not follow the angle taken by the tip but instead becomes closely associated with the outer doublet on the edge opposite the one forming the internal angle. The association is with either outer doublet 1 or 5 depending on the cirral sheet to which the cilia belong. Bars: a = 2.5 μm ; b = 1 μm .

Figure 7. Scanning electron micrographs of *Dreissena polymorpha* showing (a) portions of three gill filaments. In this low magnification image cirri are in both the flexed (F) and extended (E) positions. In the flexed position cilia from the two cirral plates form the shape of a "V" (white arrowheads) as they bend over the frontal cilia (FC) of the filament. (b) Higher magnification of the cirri in the flexed position. Note the fused body of an individual cirrus (arrow) and the "V" shape formed by the free tips (white arrowhead). The "V"-like form (white arrowhead) is observed as the free tips move over the frontal cilia (FC). The tips of the lateral cilia (L) are also visible. (c) Higher magnification with cirri in the extended position showing that the free ciliary tips extend between adjacent cirral plates forming a net-like structure. The black arrowhead indicates one such tip. The size of the spaces in the net ranges from 0.2 to 0.7 μm , with most being in the 0.4–0.5 μm range. Bars: a = 10 μm ; b = 5 μm ; c = 5 μm .

metachronal beat composed of waves of ciliary movement (about 4/s) that draw water down between the filaments and into the ostia. The lateral cilia beat in the axis of the filament. The attendant water flow appeared to be organized primarily along the length of the filament, but the location of the underlying ostia and their influence on the flow was evident. A somewhat circular current pattern over the underlying ostia was revealed by the motion of suspended fluorescent particles (data not shown). Also associated with the metachronal beat of the lateral cilia was a rhythmic movement of adjacent gill filaments. We could not determine whether the cilia were causing the filaments to move, or whether the filaments were moving rhythmically due to muscle contraction in synchrony with the ciliary beating.

Spontaneous cirral beating was more irregular than that of the lateral or frontal cilia in the isolated gills. Most often the cirri cycled rapidly and synchronously; at other times some cirri were cycling, and a few were acting independently or were motionless. The beat of the cirri was usually slower than that of the lateral ciliary beat, but we observed beat frequencies ranging from 3 to 15 beats/s in most gills. The wave form of the latero-frontal cirri was very different from that of either the frontal cilia or the lateral cilia and was based on the unique structure of these organelles (described below).

Cirral structure

An anterior-posterior cross section of the gill demonstrates the location of the cirri on the gill filaments (Fig. 1). Cirri originate from the apical end of latero-frontal cells and extend into the interfilament space (Figs. 1–3). A single latero-frontal cirrus is composed of a pair of fused ciliary sheets (or plates) both of which exit a single latero-frontal cell. A single cirral plate is shown in Figure 4. The ciliary basal bodies at the base of each cirral plate are arranged linearly, 38–42 in a row, with two parallel rows in each cell forming the base of a cirrus. Each row of basal bodies is aligned parallel to the anterior-posterior plane of Figure 1. The basal bodies of adjacent cilia in a cirral plate are tied together by electron-dense structures (Fig. 3) that connect an individual cilium with its neighbor. Each cirral cilium is connected through the electron-dense structures (Figs. 3, 5) to long intracellular rootlets that extend to the base of the cell (Fig. 3). Rootlet anchoring of cirral cilia is not uncommon for other types of cirri found in a variety of organisms (Gibbons, 1961; Sleight, 1962). No other connections distal to the basal body between the individual cilia of a cirral sheet were observed in *D. polymorpha* nor have any been reported in previous descriptions of the cirri of bivalve gills (Owen, 1974; Owen and McCrae, 1976; Gibbons, 1961).

The number of cilia forming a cirrus in *D. polymorpha*

varied but was between 38 and 42. Each cilium exits the apical side of the cell such that it becomes a component of one of the paired sheets. For most of its length, the ultrastructure of each cilium was unexceptional (Sleight, 1962): *i.e.*, a regular 9 + 2 axoneme (Fig. 5a) containing all of the normal structures, including dynein arms, radial spokes, and nexin. Midway up the length of a cirrus, each cilium in the sheet showed axonemal substructures in register (similar alignment) with its neighbors, indicating that the patterns of beating of all the cilia in a cirrus are coordinated: *i.e.*, each cirral sheet beats as a unit. This was confirmed by 'real-time' confocal observations.

Two marked differences between cirral cilia and "typical cilia" are important to cirral function (Figs. 5, 6): a distinct "hinge" region at the base of each cilium (Fig. 5a, b), and a long free apical tip region in which the number of axonemal microtubules is reduced to a small number of individual microtubules (Fig. 5c).

Each cilium in a sheet has a free tip, and the length of each cilium varies along the face of the cirrus with the shortest cilia located frontally (Figs. 4, 6). In contrast, the lengths of the free tips of the cilia are relatively constant across the entire face of the cirrus (Fig. 6a), although the diameter is progressively reduced (Fig. 5c). The initial reduction of the axoneme as it forms the free tip is abrupt (Fig. 6a, b). A shoulder region, formed by the successive reduction of the outer microtubular doublets to singlets and a shift to a more eccentric position of the central microtubular doublet, forms a distinct bulge on one side of each cilium. The shoulder region is roughly 0.9 μm in length, with the bulge forming about 0.5 μm of that length. This region has been described previously for the cirri of *Mytilus* by Owen (1974). In *Mytilus*, the region was described as having a stiffening rod associated with the free tip, and doublet #1 was indicated as being associated with the stiffening rod. In *Dreissena*, an electron-dense material connects the central microtubule doublet to outer doublet #1, but the material is not as extensive as the stiffening rod described by Owen (1974) for *Mytilus*. At the end of this abrupt shoulder region, only a single outer microtubular doublet remains, and the realignment of the central doublet to a more eccentric position almost perpendicular to the remaining outer doublet is evident. Further toward the free tip, the singlet microtubules begin decreasing in number (Fig. 5c). While the pair of microtubular doublets in each cirral cilium described above define the extended organization out to the tip, the number of other microtubules in the axoneme continued to decrease along the length of the free tips (Fig. 5c).

The presence of the shoulder region causes the fluorescent "beading" at the end of the fused cirral body observed in confocal images (Fig. 4). The nonfluorescent band between the free tips of the cirral cilia and the fused

cirral base in static laser confocal images made the cirrus in such images appear discontinuous (Fig. 4). This apparent discontinuity occurs because the conformation leaves this region specifically out of the plane necessary for fluorescent visualization and causes a lack of reflection in the proper focal plane for confocal microscopy in a single Z plane. At the shoulder, the free tips of the dorsal cirral sheet form an acute angle oriented dorsally, and the ventral cirral tips are bent ventrally.

The "hinge" region of each cirrus is located at its base. Its position is just under the microvillar layer of the latero-frontal cell, but above the cell surface, at a height about $0.2\ \mu\text{m}$ or about $1/3$ the length of the microvilli ($0.7\text{--}0.8\ \mu\text{m}$). The hinge region has multiple morphological components and is about $200\ \text{nm}$ long. The hinge region is narrower than the rest of the axoneme proper (Fig. 5b). The central doublet of the axoneme inserts onto an electron-dense basal plate of the hinge region distal to the cell surface (Figs. 5a in cross section; 5b in longitudinal section). The outer doublets are surrounded by an electron-dense material along the length of the hinge region (Fig. 5a, b). In longitudinal section, there are two electron-dense "plates" at the base of the hinge that span the axonemal region (Fig. 5b). The distal plate is thicker than the basal one and is continuous with the peripheral electron-dense material described above. These "plates" of electron-dense material are also observed in cross section (Fig. 5a). The hinge region is distinct from the junction of the basal body and the cilium, in that the outer microtubules are in the form of doublets both above and below the hinge plates (Wheatley, 1982).

Cycle of cirral movement and particle capture

A laser confocal microscope collecting images at rates from 30 to 240 frames/s allowed observation of the cirral motion in real time. When the latero-frontal cirri are extended, they project perpendicular to the filament into the interfilament space at the level of the latero-frontal cell from which they originate (Fig. 7a). The two sheets of fused cilia composing a cirrus are arranged in a dorsal-ventral orientation. The tips of the cirral cilia (CC) located on the dorsal sheet splayed out dorsally; the ventral CC tips, ventrally (Fig. 7b). The departure of the tips from the plane of the cirral body impart a feather-like appearance to the cirral pair (Fig. 7a, c). The free tips of the CC are sufficiently long that the tips of adjacent cirri form a meshwork or "net," with the spaces between the tips having submicron dimensions (Figs. 7a, c). This cirral net has been described for several other species previously (e.g., Owen and McCrae, 1976; Ribelin and Collier, 1977).

The cirrus moves to a flexed position by combining first a pivoting motion at the hinge followed by a coordi-

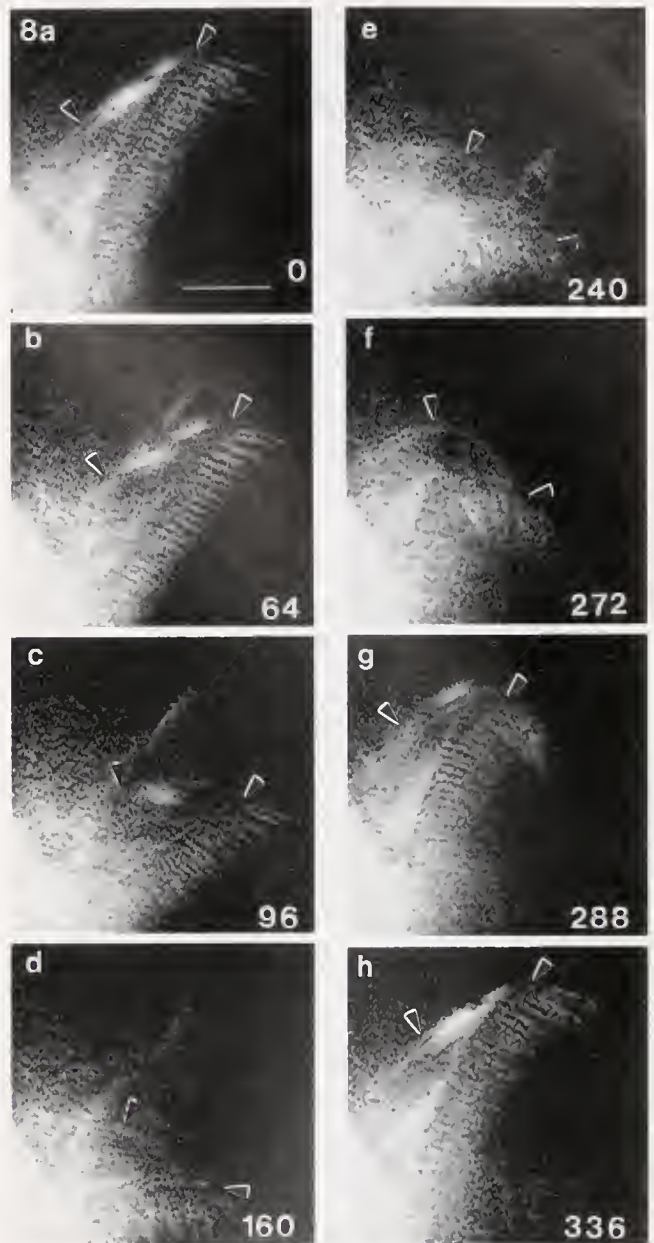


Figure 8. A series of laser confocal video images (120 frames/s) of a cirrus from the gill of a live *Dreissena polymorpha*. The focal plane is perpendicular to the dorsal-ventral axis of the filament and allows observation of one cirral plate and its free ciliary tips. These static images show one beat cycle of a cirrus. The images also contain the beat of a neighboring cirrus in the background. The first image in this series is designated time 0. Each additional image in the series shows the time in ms (lower right hand corner) elapsed from the first image. The power stroke took about 200 ms, while the recovery stroke took only 136 ms. Movement of the hinge region and the waveform beat of the cirrus is evident. The two arrowheads in each micrograph denote the mid-region of the cirral plate and the junction of the cirral plate with its free ciliary tips. Bar = $5\ \mu\text{m}$.

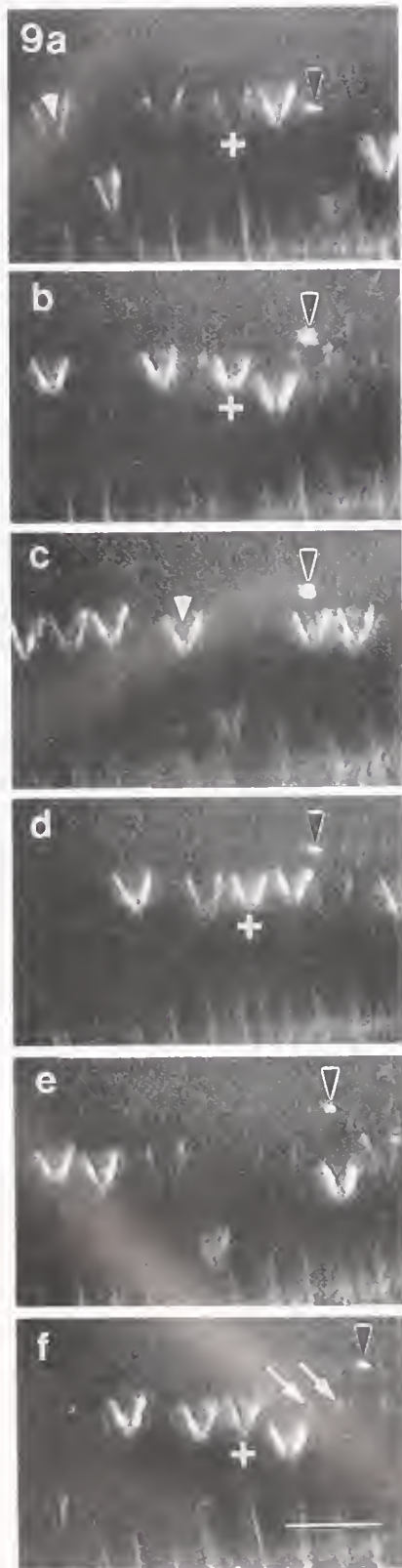


Figure 9. A series of laser confocal video images (30 frames/s) of living *Dreissena polymorpha* gill. The frontal surface of the filament is located at the top of each image. Individual cilia on this surface are out

nated curvature of its component cilia (Fig. 8). This moves the cirrus to an arched frontal position extending over the frontal surface of the filament (Fig. 7a). In some cases, flexed cirri from opposite sides of the filament extended far enough so that, together, they covered about 50% of the frontal surface of the filament. In this flexed forward projection, the cirri appeared to form open "V's" as viewed frontally with scanning electron microscopy (Fig. 7a, b).

A time series captured by laser confocal microscopy at 120 frames/s is shown in Figure 8. The entire cycle for this particular cirral beat was 336 ms. The images were chosen to represent the beat cycle, and the time elapsed from the first image is indicated on each of the subsequent images. The power stroke took about 200 ms (Fig. 8a–d) while the recovering stroke took another 136 ms (Fig. 8e–h). The movement on the basal hinge described above and the gradual curvature of the ciliary bodies during the flexing movement of the cirrus can be seen. The more rapid whip of the cirral cilia back toward the extended position, as well as the movement on the basal hinge, can be observed. Cirri often move faster than the one presented in this time series, but the beat process and the ratio of time spent in each component of the cycle

of the plane of focus. The cirri on one side of the filament are visible. The beat of these cirri resulted in a flexion into the plane of focus and over the frontal surface and an extension back and slightly to the left out of the focal plane into the interfilament space. The free ciliary tips of each cirrus form a bright V (white arrowhead; see Fig. 7 a, b for comparison) as the cirrus is flexed over the front of the filament. The width of the open face of these V's is about $2\ \mu\text{m}$. The dark space below the bright V's is the interfilament space, and the extended cirri drop down and out of the visible plane. The fluorescent linear structures at the base of each image are the fused plates of cirri associated with the adjacent filament located below the bottom of each image. The fluorescent dot (black arrowhead) on each image is a $0.75\ \mu\text{m}$ fluorescent head, one of many that were added to the preparation and allowed to move across the gill. In (a) the particle (black arrowhead) was free and moving below the front of the filament. In (b) a cirrus was flexing into the plane pushing the particle (black arrowhead) up onto the frontal surface of the filament. In (c) the cirrus was fully flexed and the particle was on the filament. In (d) the particle remained on the frontal surface as the cirrus with which it had interacted extended. In (e) the adjacent cirrus nears the particle as it moves into a flexed position. In (f) the cirri near the particle have extended out of the focal plane. In both (e) and (f) the particle moved to the right along the frontal surface. In this time series the velocity of the particle along the frontal surface of the filament was $25\ \mu\text{m/s}$. This time series also indicates the beat pattern of a group of eight to nine adjacent cirri. For reference, a single cirrus is identified (+) throughout the time sequence on the images where it is visible. The beat pattern was such that some cirri were flexing while others were extending. All of these cirri were beating rapidly (averaging about 15 beats/s for an individual cirrus). The movement of the particle along the frontal surface was due to the beat of the frontal cilia, which were not, however, in the focal plane of this set of images. Finally, a few of these images suggest the presence of the pro-lateral frontal cilia (arrows, Fig. 9f). Bar = $5\ \mu\text{m}$.

remain similar for faster-beating cirri. The beat pattern observed here is similar to that described by Dral (1967, 1966) for the cirri of *M. edulis*.

Figure 9 is a set of static images from a time series captured by confocal microscopy; it shows a representative interaction between a $0.75\ \mu\text{m}$ fluorescent particle and a cirrus. The interval between each frame is 33 ms, except that between (e) and (f) which is 66 ms. The identified particle was moving freely before the initial frame (Fig. 9a); then it interacted with a cirrus and was pushed toward the frontal surface of a filament (Fig. 9b, c). Once the particle was pushed up onto the front of the filament (Fig. 9d), it was quickly moved along the frontal surface. In the 100 ms that elapsed between Figures 9d and f, the particle moved $2.5\ \mu\text{m}$ along the frontal surface. This time series is typical of our observations and indicates the capture and movement of such particles to the frontal surface by an individual cirrus. In cases where the first sweep of a cirrus did not successfully move the particle onto the frontal surface, the particle often interacted with the same or several other cirri until it was pushed far enough onto the frontal surface that it began moving along that surface. Similarly, particles that interact with an extended cirrus were pushed forward with the flexion of the cirrus to the same position as seen in the example (Fig. 9).

The most lateral of the frontal cilia (pro-lateral-frontal cilia) were also observed to move into the open "V" face of the cirrus with a beating motion that would appear to aid in the transfer of particles from the cirral face onto the frontal cilia (Fig. 9). The branches of the "V" are composed of the aggregated ventral and dorsal CC tips, and the base of the "V" is composed of the dorsal and ventral faces of the fused sheets. The open face of the "V" is about $2\ \mu\text{m}$ across.

Discussion

The results of this study clearly indicate that cirral structure, position, and motion in *Dreissena polymorpha* create a "capturing net," and that the net is active in particle capture. This does not exclude a role for the branchial water currents created by cirral movement (Jørgensen, 1981; Nielsen *et al.*, 1993); it simply indicates that cirral "net" filtration is indeed an important component of small particle capture by the gill in *D. polymorpha*. Atkins (1937, 1938) described with remarkable accuracy the existence and structure of latero-frontal cirri on bivalve gills from many different lamelli-branch families. She recognized that these cirri are formed from multiple fused cilia. Cirral structure has subsequently been described for a number of different species using ultrastructural techniques. The species

most often studied to date have been *Mytilus edulis* and *Crassostrea virginica*. *C. virginica* has a plicate, heterorhabdic gill. It has rather small cirri, with each cirral plate containing less than 20 cilia, and with cirral cilia slightly more numerous on ordinary filaments than those on principal filaments (Ribelin and Collier, 1977). The cirri near the marginal groove of these filaments are very small, composed of only 3–4 cilia per plate (Ribelin and Collier, 1977). In contrast, *Mytilus edulis* has a filibranch gill. The cirri of *M. edulis* have been extensively described and are rather complex, containing approximately 30 cilia per plate (Gray, 1922; Moore, 1971; Owen, 1974; Owen and McCrae, 1976; Jones *et al.*, 1990). Both of these species demonstrate cirral nets formed by free ciliary tips in ultrastructural studies (Moore, 1971; Owen, 1974; Owen and McCrae, 1976; Ribelin and Collier, 1977).

Atkins (1938) subdivided the bivalves into the Macro-ciliobranchia and the Microciliobranchia based on the presence or absence of cirri, respectively. Interestingly, because of the relatively small size of cirri in the Ostreidae, Atkins included this family in her Microciliobranchia. Owen and McCrae (1976) in their discussion also note that the size and structure of the cirri on bivalve gills vary extensively with species and gill type. Owen and McCrae (1976) further relate differing particle capture mechanisms to the two groups (placing Atkins's outlier the Ostreidae into the Macro-ciliobranchia), suggesting that current flow and mucus are the dominant mode for particle collection in the Microciliobranchia, and that actual particle capture occurs in the Macro-ciliobranchia. The complexity of cirral structure in *D. polymorpha* is more similar to those found in *M. edulis* than those in *C. virginica*.

Dreissena polymorpha has high particle filtration rates compared to other freshwater mussels (Jørgensen *et al.*, 1984; Kryger and Riisgård, 1988; Bunt *et al.*, 1993; Silverman *et al.*, 1995), especially for particles smaller than $2\ \mu\text{m}$. The location, orientation, and dynamics of the latero-frontal cirri on the gill of *D. polymorpha* suggest that they are responsible for particle capture. Cirral motion and interaction with fluorescent beads, as observed by confocal microscopy, confirm cirral involvement in the particle capture process. The "capture net" that forms across the interfilament space by cirri in the extended position is readily apparent with SEM. The size of the spaces formed by the net are in the right order of magnitude ($0.2\text{--}0.7\ \mu\text{m}$) to allow the capture of particles attributed to *D. polymorpha* (Morton, 1971). Whether capture is by actual trapping within the "net" or whether the net is behaving more like a "solid" capture sheet remains to be determined (Purcell, 1977).

The hinge mechanism on the cirrus allows it to assume, alternately, an extended or flexed orientation rel-

ative to the position of the latero-frontal cells on which it originates. The motion of the cirrus suits both an initial capture event and the delivery of captured particles onto the frontal cilia for further processing. The cirral hinge region has been described previously as the ciliary neck region of unknown function, but showing specialized membrane structure by freeze-fracture examination (Good *et al.*, 1990). The static images of the SEM indicate the potential, and the dynamic images of real-time laser confocal microscopy demonstrate the actual process of cirral movement as it occurs.

We saw no evidence, either in live isolated gills or SEM samples, that mucus contributes in a major way to this component of particle capture. There are no obvious mucus cells located on the gill filament except at the base of the filament near the external ostia. No mucus production was evident in the vicinity of the cirri. There does not appear to be a major requirement for mucus participation in the capture of small particles in *D. polymorpha*, at least in the isolated gills observed in this study. Mucus cells are present below the level of the lateral ciliated cells and are prominent at the entry of the water canal into the central water channel and along the water channel epithelium. Mucocytes are also prominent at the dorsal margin leading to the suprabranchial chamber. Mucus clearly plays a role in particle transport (Beninger *et al.*, 1992; Ward *et al.*, 1993) and food processing in a wide variety of bivalves. In addition, our preliminary studies on excised unionid gills indicate that mucus is conspicuously associated with cirri in several species (unpubl. obs).

Although we have examined only one species to date in detail, so extensive speculation is not warranted, the mechanism described here for capture of small particles ($\leq 2 \mu\text{m}$) may explain the feeding data presented in several studies. These studies have shown that species with complex cirri are more efficient in removing small particles than those species with less complex cirri, or with no cirri at all (Owen and McCrae, 1976; Møhlenberg and Riisgård, 1978; McHenry and Birbeck, 1985; Riisgård, 1988).

The mechanism of particle capture has been debated, yet there may not be a single mechanism, but rather a suite of mechanisms contributing to particle capture by bivalves with widely differing demibranch structures (Ward *et al.*, 1993). Jørgensen (1981, 1982, 1989) indicates that these cirri along with the other ciliary components of the filament create a set of three-dimensional currents that act in concert to entrain particles in the water column. The hypothesis is based on various current measurements and interpretation of other studies indicating that the cirral mesh should trap all particles above a certain size. This hypothesis has not been confirmed by experimental observation (Vahl, 1972; Møhlenberg and Riisgård, 1978). Our results indicate that cirri comprise

a moving, dynamic mesh that will not be capable of capturing every particle, particularly at the lower limits of the mesh size. When a single cirrus is bent frontally it would not be in position to exert a sieve effect. This suggests that the trapping efficiency of the cirri cannot be 100%, particularly at particle sizes approaching the lower limits of their capturing ability. Our interpretation agrees with the observations and calculations of Silvester and Sleight (1984; Silvester, 1988). Our observations coincide with the experimental data of Owen and McCrae (1976) indicating that the mesh size of the cirral "net" in a few selected species of marine bivalves is roughly correlated with the size of particles filtered by a particular species. Silvester and Sleight (1984) provided physical calculations indicating that the size range and quantity of particles actually trapped by gills under various experimental conditions are consistent with the cirral mesh size in *Mytilus*. Comparisons of several marine species by Riisgård and colleagues suggest that larger, more complex cirri may be associated with the ability to trap particles on the order of $2 \mu\text{m}$ (Møhlenberg and Riisgård, 1978; Riisgård, 1988). McHenry and Birbeck (1985) indicate that marine species with complex cirri (*Mytilus edulis*) are better able to clear *Escherichia coli* than bivalves with less complex cirri (*Ostrea edulis*), whereas a species with no latero-frontal cirri (*Chlamys opercularis*) is unable to capture bacteria. Silverman *et al.* (1995) have also shown that in three species of freshwater bivalves, the clearance of *E. coli* is correlated with the structural complexity of the gill cirri.

By describing the "capture net" of *D. polymorpha* with mesh spacing between 0.2 and $0.7 \mu\text{m}$, we raise the issue of fluid and particle movement that may be more affected by viscosity than inertial forces (*i.e.*, low Reynolds number effects). In several other species (echinoderm larvae, rotifers, copepods), studies on particle capture by structures with small net spacings have indicated that such structures act more like solid paddles moving fluid than as actual sieves capturing particles (Strathmann, 1971; Strathmann *et al.*, 1972; Koehl and Strickler, 1981; Strickler, 1982). The size range and structure of the cirri clearly suggest low Reynolds number fluid mechanics (Rubenstein and Koehl, 1977). Under steady state conditions, mathematical modeling and experimental testing of the capture mechanism of nets or sieves of this type indicate that they would act more like paddles than nets (Rubenstein and Koehl, 1977; Shimeta and Jumars, 1991). However, for the gill of *Mytilus edulis*, Nielsen *et al.* (1993) suggest, from partial filament model systems, that the interfilamental area of the gill is not strictly in a steady flow state due to the velocity differences in this region caused by the activity of the ciliary pumps. Shimeta and Jumars (1991) suggest that under nonsteady state conditions particle movements and

interaction with filter elements may vary considerably from that predicted by steady flow modeling.

Confocal microscopy confirms that cirri trap or deliver 0.75–1 μm fluorescent beads to the frontal surface of the gill. The movement of a 0.75 μm particle by a cirrus onto the frontal surface of the filament is shown in the time-lapse images captured using laser confocal microscopy (Fig. 9). These images by themselves do little to confirm whether the delivery mechanism is strictly hydromechanical or whether some particle contact with the cirri occurs. Given the unsteady flow environment, only exacting measurements of fluid velocities at the cirral sites themselves, identification of particle trajectories, and measurement of shear and drag forces will allow the exact mechanism of cirral interaction with particles to be determined. What does appear to be clear from the data presented in Nielsen *et al.* (1993) for *Mytilus* and for our confocal observations of *Dreissena* is that a close interaction between gill cirri and particles results in particle capture.

We have observed here that cirri can capture particles and that isolated capture events can be observed by confocal microscopy. The observations have only been made in a single species, but they demonstrate the potential role that cirri can play in particle capture by bivalves. What is not demonstrated by these observations is whether particle capture by cirri is the predominant mechanism of particle capture or whether other capture mechanisms provide for more particle capture by a gill than the cirral mechanism. The answer to this question is likely to vary with species, and particularly to variations in cirral structure and overall gill structure. For a given species, the relative roles of hydrodynamics and the mechanics of inertia and viscosity in suspension feeding events will therefore depend on structure, assortment, and location of the locomotory (ciliary) elements, the mesh size of cirral nets, the diameters of the pores, and the sites and amount of mucus release. The assumption that modest differences in cirral structure are not important to function because they are all acting similarly as paddles, fails to take into account that overall gill function in each bivalve is determined by a suite of variable characters whose functional interrelationships are crucial to the mechanisms of particle capture.

Observation of the intact, functioning gill within the pallial cavity is clearly the optimal way to account for all forces and movements that occur in that complex fluid environment. Endoscopy has provided the data for the particle transport process (Ward *et al.*, 1993), but until a resolution of about 0.5 μm is achieved, this method alone will not provide the information necessary to address the issue of small particle capture by the gills of bivalves. Laser confocal microscopy does allow real-time observation of interactions between cirri and small particles, but requires excision of the gill. Continued com-

parative studies of gill musculature, motile organelle dynamics, and the architecture of water canals and channels is required for a more complete understanding of particle capture during water movement through and across the gill.

Acknowledgments

The authors thank Julie Cherry and Ron Bouchard for their outstanding technical assistance. We thank T. R. LeBlanc at Dow Chemical Company, Plaquemine, Louisiana, for helping us collect zebra mussels. Diondi Lessard participated with support from a grant to improve undergraduate education from the Howard Hughes Medical Institute to LSU. Finally, we give special tribute to S. J. Nichols and P. G. Beninger for sharing their insights with us. The Life Science Microscopy Facility at LSU provided the equipment necessary for this study. A short video is currently being placed on the world wide web at <http://www.chem.lsu.edu/~wwwzool/welcome.html>, for those who wish to see the time series of particle capture in motion. The work was supported by Louisiana Sea Grants R/ZM-1-PD and NA46RG0096 project R/ZMM-1, and NSF DCB90-17461.

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Finding Food: Neuroethological Aspects of Foraging

Proceedings
of a symposium
in honor of
Vincent Gaston Dethier
(1915–1993)

University of Massachusetts
at Amherst

6–8 October 1995

Acknowledgments

The symposium was supported by the Vice Chancellor of Research, Graduate Studies and Economic Development, the Dean, College of Natural Science and Mathematics, the Departments of Biology and Entomology, and the programs in Neuroscience and Behavior and Organismic and Evolutionary Biology at the University of Massachusetts, Amherst; by grant #IBN-9513884 from the National Science Foundation; and by an anonymous donor.

Finding Food: Neuroethological Aspects of Foraging. Introduction to the Proceedings of a Symposium in Honor of Vincent Gaston Dethier (1915–1993)

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This collection of papers results from a symposium dedicated to Professor Vincent G. Dethier, a pioneer in studies of sensory physiology and feeding behavior. Throughout a remarkable six decades of research and writing, Vince Dethier's interests focused upon the sensory and physiological mechanisms of feeding in caterpillars and flies. His earliest research addressed problems of host-plant selection; this work led him into the study of the chemical sense of caterpillars and from there, launched him into the general area of chemoreception. While his research primarily addressed consummatory aspects of feeding, the appetitive components, how animals find their food, always contributed to the development of his ideas.

Vince was a man of enormous erudition who brought his breadth of interests into every aspect of his approach to biology. It was our privilege to enjoy this erudition when he joined the biology department at the University of Massachusetts. To honor him we wanted to design a symposium that mirrored the depth and breadth of his scholarship. We decided upon foraging as a unifying theme, one that provided a conceptual framework while still drawing on a wide variety of disciplines. Kamil and Sargent (1981) have stressed the importance of studies of foraging in ethology, psychology, ecology, and anthropology. Certainly it is an area that draws on locomotion, sensory processes (sensitivity, integration, and interpretation), hormonal and other physiological states, learning and memory, and all possible combinations and

permutations. The symposium's approach to foraging behavior was primarily sensory, integrating mechanistic, functional, and modeling perspectives.

Analyses of approaches to animal behavior started formally with Tinbergen's formulation of four aspects of behavior study: mechanism, function, development, and evolution (Tinbergen, 1963). Since that time there have been a number of shifts in conceptual approaches to behavior. Huntingford (1993) points out that early ethological studies concentrated on causation at the expense of function, but were replaced over the last 20 years by an "excessive concentration on behavioral function at the expense of the study of mechanisms." This shift in interest has led to the development of sophisticated models for which there is often insufficient mechanistic support. A tension that exists between empirical and modeling studies of behaviors such as foraging is made explicit by Bell (1991) in the preface to his book, *Searching Behaviour*. He rebels against the seductive hold of mathematical models:

. . . the intent is to offer a comprehensive view of the mechanistic side of searching (or foraging) so as to balance the current emphasis of books on mathematical and functional models. . . . [T]he pendulum needs to swing back to studies of how animals behave, and that maybe in so doing models will become valuable again in driving experimentation.

Recent publications (Huntingford, 1993; Real, 1992, 1994; and Hughes, 1993) point out the strengths and weaknesses of our present understanding of behavior. They also point out the need to integrate all that we have learned so far into more realistic models. Such an inte-

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gration is becoming a real possibility. Two major constituents, behavioral ecology and sensory ecology, have recently benefited from more focused attention. Behavioral ecology—why behavior is adaptive—crystallized with Krebs and Davis' book (1978), and Gross (1994) considers it to have matured as a discipline in 1986. Sensory ecology—tracing the flow of sensory information within an ecological community—is beginning to mature with the publication of Dusenbery's (1992) book of the same name.

The following papers integrate neuroethology, behavioral ecology, and sensory ecology. The majority address questions of how animals navigate and find food in informationally complex or rapidly (micro- to nanosecond) changing environments. They ask how animals might perform sophisticated discrimination tasks. Laurent and his co-workers (1996), for example, ask how animals might have unique neural representations of specific complex odors. Smith (1996) asks how they can learn these odors and generalize the memory so that a rose is always a rose, while Atema (1996) develops hypotheses to suggest how a lobster might navigate through a constantly shifting odor landscape. Simmons *et al.* (1996) analyze how bats can keep track of prey moving rapidly and evasively amongst branches and leaves, and show that the bats detect submicrosecond changes in echo signals. Dusenbery (1996) describes nematodes that move along a temperature gradient as shallow as 0.001 °C/cm to get to a region in the soil where they are likely to encounter the plant roots they need. Laurent *et al.* (1996), Simmons *et al.* (1996), and Kawasaki (1996), each group studying a different sensory system, all find that the whole is much greater than the sum of its parts. Discriminating ability can be predicted from the integrated activity of several central neurons, whereas the activity of a single neuron cannot be extrapolated to explain the observed behavior.

The other papers ask how animals might cope with longer term environmental changes—changes during feeding, from season to season, or through metamorphosis. Stephens (1996) develops a model to describe how digestion might control patterns of eating; animals are unlikely to forage while they are satiated. Jacobs (1996) links cognitive neurobiology and behavioral ecology in examining how small mammals and birds cope with the reduced energy sources available during the winter. She describes remarkable changes in the hippocampus of squirrels, voles, pigeons, and chickadees that correlate with the varied behavioral strategies of these animals. Weeks and Woods (1996) have examined both short- and long-term neural changes in the tobacco hornworm caterpillar. This animal has a reflex, the proleg withdrawal reflex, that surely reduces its chances of being for-

age because the prolegs cling more tightly to the substrate when mechanoreceptor hairs are stimulated. Weeks and Woods (1996) have traced neurophysiological and anatomical changes in the neural circuitry of this reflex during habituation and also during metamorphosis.

All these papers demonstrate how remarkably subtle and flexible nervous systems must be, how far we can understand and analyze these subtleties, and how important is the integration of the activity of individual neurons. The neuroethological approach and the strong basis in sensory physiology of these papers echo fundamental aspects of Dr. Dethier's contributions and expertise.

A more complete introduction to Vince's life and work can be found in the memorials written by his former students and colleagues Alan Gelperin (1994) and Frank Hanson, Louis Schoonhoven, and Ron Prokopy (1995).

Acknowledgments

Our work is supported by a grant from the Whitehall Foundation.

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Learning, Memory, and Neural Networks: Introduction

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Learning and memory functions in animals, particularly insects, were enduring themes in the research program of Vincent Dethier. He considered how behavioral plasticity contributed to the fitness of his experimental subjects and what general principles were illuminated by this plasticity (Dethier, 1966). With the keen eye of a field naturalist he posed learning questions directly relevant to the *Umwelt* of his favorite subjects, lepidopterous larvae (Dethier, 1988). It is therefore particularly fitting in our symposium honoring Vincent Dethier to consider how caterpillars of *Manduca sexta* modify a neural circuit for larval proleg withdrawal by nonassociative learning and by remodeling during metamorphosis. Detailed cinematographic analysis of larval feeding led to identification of the proleg withdrawal response as a candidate for studies of behavioral plasticity amenable to detailed cellular analysis. Weeks and Wood (1996) show that the effects of steroid hormones on remodeling *Manduca* motoneuron dendrites relate directly to similar phenomena in avian and mammalian brains. The power of the analysis in *Manduca* is revealed in the level of cellular resolution obtained in specifying the mode of action of steroid hormones on dendritic domains of identified neurons with clear links to behavior.

The central processing of chemosensory information is another arena in which Vincent Dethier made a continuing series of seminal contributions (Dethier, 1941, 1990). Within olfactory systems in creatures as diverse as locusts, slugs, frogs, fish, and mammals, the first central network engaged in olfactory processing shows spontaneous or odor-triggered oscillations (Gelperin *et al.*, 1996). The computational function of these coherent oscillations shared among a distributed population of cen-

tral interneurons responding to a common odor stimulus is explored by Laurent and colleagues (1996) with unprecedented clarity using multiple *in vivo* intracellular recordings from antennal lobe and mushroom body neurons in the locust. The several possibilities considered for how shared oscillations augment the odor-recognition ability of locust central networks have relevance across multiple sensory modalities as well as wide phyletic bounds. Another function for olfactory oscillations may be to enhance detection of very weak odors by having the input sensitivity of the oscillating circuit maximal at a particular phase of the oscillation cycle; this possibility is suggested by the differential phase sensitivity of the *Limax* olfactory network to olfactory nerve shock and by a mathematical model of the bursting *Aplysia* neuron R15.

Among insect scholars, the honey bee is preeminent (Menzel and Müller, 1996). With odorants as conditioned stimuli and feeding stimulants such as sucrose as the unconditioned stimulus, Smith (1996) describes a variety of higher-order conditioning phenomena in honey bees. These experiments address questions of how sensory input from odor mixtures may be processed to maintain perceptual constancy in the face of natural variations in odor intensity and odor composition (Hopfield, 1995). A computational model of odor processing and odor learning in the honey bee has been developed that will be very helpful in this effort. The model is derived from established morphological, physiological, and behavioral data and makes explicit testable predictions about local changes in the ratio of excitation and inhibition in the bee's antennal lobe as a result of odor learning (Linster and Masson, 1996). As sites of synaptic plasticity involved in odor learning are identified in the honey bee brain, it will be possible to determine whether the biochemical machinery for long-term memory storage identified in studies of fly, slug, and mouse brains (Gelp-

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erin, 1996) operates in honey bees. A unity of memory-storage mechanisms between insects and mammals would be no surprise to Vincent Dethier.

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Short- and Long-Term Modification of Reflex Function During Learning and Metamorphosis in *Manduca*

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However much one may disparage the stolid abdominal crawling behavior of caterpillars, the fact remains that they possess probably the most versatile abdomens in the animal kingdom. . . . The abdomen is to the caterpillar what arms are to a person.

. . . caterpillars are such schizophrenic beasts. At one and the same time they must be perfect caterpillars and potential moths. They must be prepared to meet all the challenges that life presents to a slow-moving, earthbound grazer while beginning the construction of the fragile furred body and gossamer wings that will offer them the freedom of the air.

—Dethier (1980)

Introduction

In his book, *The World of the Tent-Makers*, from which the above quotations are taken, V. G. Dethier chronicles the lepidopteran life cycle in an absorbing blend of lyric prose, philosophy, and astute observation of animal behavior. As detailed in this book, the insect's life is marked by countless challenges and opportunities to which the nervous system must respond in an adaptive manner. Although the basic neural circuits underlying many behaviors are preassembled in the egg, these behaviors must be modified to accommodate the exigencies of the real world. Furthermore, during metamorphosis, the neural circuits for larval behaviors must be replaced with those for pupal and adult behaviors. This review describes our investigations into how the nervous system strikes a balance between constancy and flexibility in generating behavioral output.

Our experimental animal is the tobacco hornworm, *Manduca sexta*, which transforms from egg to moth in

approximately 6 wk. *Manduca* has proven to be ideal for investigating the neural mechanisms of behavior, as well as how hormones—the ecdysteroids and juvenile hormone (JH)—reorganize the nervous system during metamorphosis (reviewed by Weeks and Levine, 1992). This review focuses on a neural circuit that mediates a simple defensive withdrawal reflex of the larval proleg of *Manduca*, and describes how reflex function is modified in two contexts: during the dismantling of the circuit at pupation and during nonassociative learning in the larval stage. The signals for these two forms of plasticity differ, as do the associated electrophysiological changes that underlie the behavioral changes. Even in this simple reflex circuit, a variety of neural mechanisms contribute to behavioral modifications that occur on time scales from seconds to days and permit the insect to interact adaptively with the world around it.

The Proleg Withdrawal Reflex

Manduca larvae have 8 pairs of legs: 3 pairs of segmented legs on the thorax and 5 pairs of prolegs on the abdomen (see Fig. 2). Our studies have focused on the prolegs located on abdominal segments three through six (A3–A6). Returning to Dethier's quotation above, if the caterpillar's abdomen is like a person's arms, then the prolegs are the hands on those arms: they are used to reach for and grasp objects, to crawl, and to sense disturbances. The tip of each proleg bears an array of mechanosensory hairs, the planta hairs (PHs; Weeks and Jacobs, 1987). Stimulation of the PHs evokes ipsi- or bilateral retraction of the prolegs in the stimulated segment—the proleg withdrawal reflex (PWR; Fig. 1A). The major features of the neural circuit for the ipsilateral PWR have been identified (Fig. 1B): the sensory neurons that innervate PHs excite proleg retractor motoneurons *via* mono-

Received 30 November 1995; accepted 19 March 1996.

This paper was originally presented at a symposium titled *Finding Food. Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

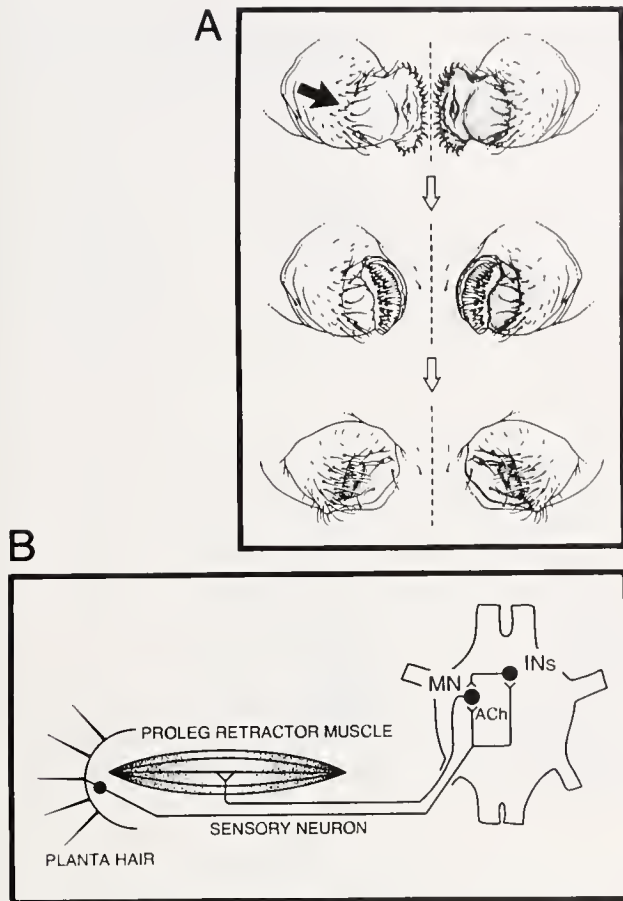


Figure 1. PWR behavior and neural circuit. (A) The PWR. A pair of prolegs is shown in ventral view with the ventral midline marked by a dashed line. Tactile stimulation of PHs (filled arrow) evokes proleg withdrawal, as shown in the successive drawings (open arrows). (B) PWR circuit. The proleg tip (left), a proleg retractor muscle (middle), and the ganglion of the same abdominal segment (right) are shown schematically. Neurons are indicated by filled circles. Each PH is innervated by a single sensory neuron with a cell body in the proleg epidermis and an axon that projects to the ganglion. PH sensory neurons excite ipsilateral PPR and APR motoneurons (MN) via monosynaptic, cholinergic (ACh) synapses, and polysynaptic pathways through interneurons (INs). The diagram shows the neural circuit for the left proleg only.

synaptic, cholinergic connections and by polysynaptic pathways involving unidentified interneurons (Weeks and Jacobs, 1987; Peterson and Weeks, 1988; Trimmer and Weeks, 1989, 1991; Jacobs and Weeks, 1990; Sandstrom and Weeks, 1991; Streichert and Weeks, 1995). The interneuronal pathway includes excitatory and inhibitory interneurons, but the excitatory components predominate. All of the necessary neural components for the PWR are located in the ganglion of the same segment as the stimulated proleg. Studies of plasticity in PWR function have focused on two identified motoneurons, PPR and APR (Fig. 2), which innervate the principal planta retractor muscle (PPRM) and the accessory

planta retractor muscle (APRM). Each proleg has one PPRM and one APRM. One PPR innervates each PPRM, whereas each APRM is innervated by a pair of similar APRs (Weeks and Truman, 1984; Sandstrom and Weeks, 1996). These motoneurons and muscles represent the output pathway of the PWR reflex circuit.

Long-Term Modification of the PWR Circuit During Metamorphosis

Dismantling of the PWR circuit

Over the final few days of larval life, the prolegs collapse and stiffen, most of their retractor muscles degenerate (see below), and the PWR can no longer be elicited. Loss of the behavior results in part from the weakening of central excitatory pathways in the circuit, as indicated by a developmental decrease in the ability of electrical stimulation of the proleg sensory nerve to evoke reflexive firing in proleg motoneurons (Jacobs and Weeks, 1990). The decreased excitation could involve changes in sensory neurons, interneurons, motoneurons, or all three, but a previous finding that the dendrites of PPR and the APRs regress dramatically over the same period that the PWR disappears (Fig. 2) suggested that they might be a key locus of change. As reviewed elsewhere (Weeks *et al.*, 1996), dendritic regression is triggered by a prepupal peak of ecdysteroids in the hemolymph.

Because dendrites are the sites of synaptic input, we hypothesized that the regression of motoneuron dendrites would weaken their inputs from sensory neurons and interneurons. To test this idea, we stimulated the proleg sensory nerve while recording the amplitude of the compound excitatory postsynaptic potential (cEPSP) evoked in PPR and the APRs. The cEPSP reflects both the mono- and polysynaptic components of the PWR. During the larval-pupal transformation, cEPSP amplitude decreased by greater than 50% despite an increase in motoneuron input resistance (Jacobs and Weeks, 1990; Streichert and Weeks, 1995). To better define the contribution of different neurons to this decrease, we took advantage of a unique characteristic of *Manduca*: the ability to manipulate the levels of ecdysteroids and JH to generate *heterochronic mosaic* neural circuits composed of neurons in different developmental states (*e.g.*, Levine *et al.*, 1989). It is then possible to determine whether developmental changes in particular neurons are necessary or sufficient for the developmental change in circuit function. For this experiment, we generated heterochronic mosaic insects in which the PH sensory neurons on one proleg were retained in their larval form while the central components of the PWR circuit underwent pupal development. Notably, motoneuron regression in mosaic hemisegments was the same as in unmanipulated controls. In the mosaic hemisegments, cEPSP amplitude

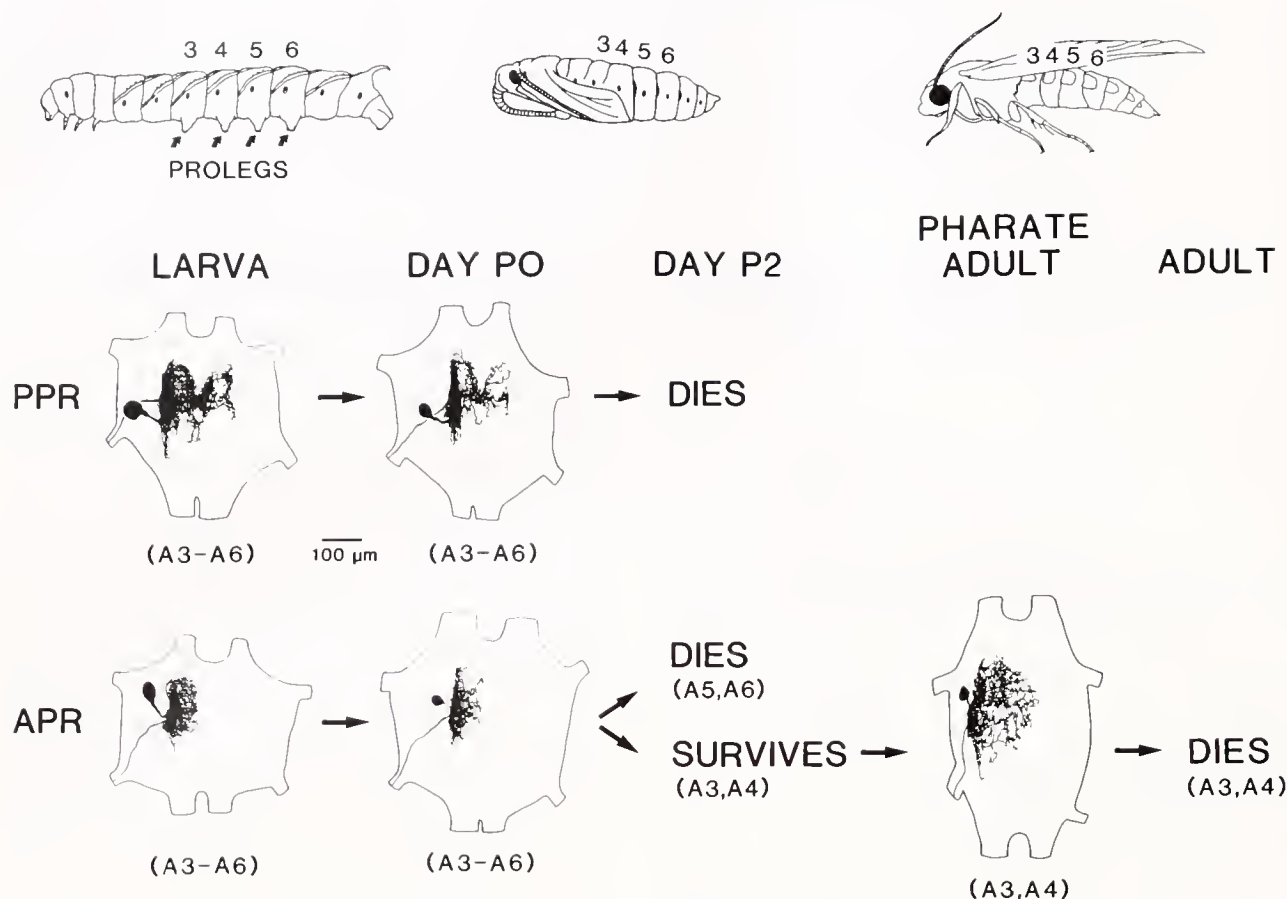


Figure 2. Life history of proleg motoneurons. (**Top**) Drawings show a larva, pupa, and adult *Manduca* with abdominal segments 3, 4, 5, and 6 indicated. (**Bottom**) Camera lucida drawings of cobalt-stained PPRs and APRs are shown on day L3 (final instar larva before the onset of metamorphosis), day P0 (first day of pupal life), day P2 (third day of pupal life), pharate adult (the final day of pupal life), and in the adult moth several days after emergence. The segmental locations of the neurons are indicated in parentheses. PPRs in all segments regress by day P0 and die by day P2. APRs in all segments regress by day P0, but only those in segments A5 and A6 die by day P2. The APRs that survive in segments A3 and A4 show dendritic regrowth during the pupal stage and die after adult emergence. Data from Weeks and Ernst-Uttschneider (1989).

decreased to the same extent as in normal pupae, suggesting that metamorphic changes in PH sensory neurons are *not necessary* and metamorphic changes in central neurons (motoneurons and interneurons) are *sufficient* for the weakening of excitatory pathways in the PWR circuit (Jacobs and Weeks, 1990; Streichert and Weeks, 1995).

One limitation of these experiments was that the cEPSP is a mixture of mono- and polysynaptic inputs to the motoneurons and hence does not reveal how individual synapses change during metamorphosis. To test more explicitly the hypothesis that dendritic regression disconnects synaptic inputs to the motoneurons, we measured the amplitude of monosynaptic EPSPs produced in APR by individual PH sensory neurons in larvae and heterochronic mosaic pupae. At both stages,

EPSP amplitude was correlated with the position of the PH on the proleg (Fig. 3A), but the key finding was the EPSP amplitude decreased significantly in all regions of the PH array during the larval-pupal transformation (Fig. 3B). Analysis of EPSP shape indices indicated that the decreased EPSP amplitude in pupae resulted from a decrease in synaptic current, consistent with a reduction in the number of synaptic contacts between the sensory neurons and motoneurons as the motoneurons regress (Streichert and Weeks, 1995).

In summary, these experiments revealed a specific mechanism that contributes to the dismantling of an outmoded neural circuit at the entry into a new life stage: the hormonally driven regression of motoneuron dendrites “sheds” sensory synapses that constitute the input pathway for the reflex. In a broader context, steroid hor-

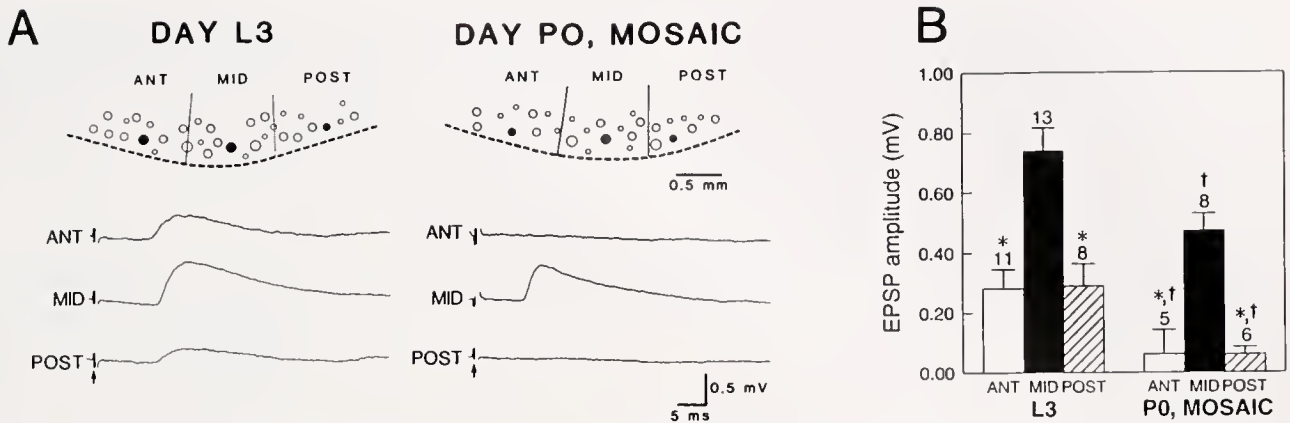


Figure 3. Developmental changes in synaptic connections between PH sensory neurons and APR. In (A), the drawings at top are maps of sockets (circles) corresponding to individual PHs in the PH array. Dashed line indicates the distal border of the PH array, and dotted lines indicate anterior (ANT), middle (MID), and posterior (POST) regions. Filled circles indicate the sockets stimulated (at 1 Hz) to produce the EPSPs shown below. Traces show signal-averaged responses recorded in an APR while stimulating the indicated sensory neurons on day L3 (left) and, in another preparation, on day P0 in a mosaic hemisegment (right). Arrows indicate stimulus artifacts. (B) Comparison of EPSPs on days L3 and P0. The mean amplitude ($\pm$ SEM) of EPSPs evoked in APRs by PH sensory neurons located in the three regions of the PH array are shown on day L3 and in day P0 mosaics. The number of PH sensory neurons tested is indicated above each bar. On both days of development, EPSPs produced by anterior and posterior PH sensory neurons were significantly smaller than those from middle sensory neurons (* indicates $P < 0.001$). In day P0 mosaics, mean EPSP amplitude was significantly smaller than on day L3 in all three regions of the PH array († indicates $P < 0.001$). Data from Streichert and Weeks (1995).

mones cause simultaneous changes in neuronal structure and behavior in many animals (e.g., Forger and Breedlove, 1987; Woolley and McEwen, 1993; Smith *et al.*, 1995), but it is typically difficult to test whether the relationship is causal or merely correlational. At present, the PWR of *Manduca* is the best-documented example in which a causal relationship has been demonstrated using electrophysiological methods. Progress is also being made in a number of other systems, both vertebrate and invertebrate (reviewed in Weeks and Levine, 1995).

Fates of leftover components of the PWR circuit in pupae

What happens to members of the PWR circuit after their larval function is obsolete? The PH sensory neurons die (Jacobs and Weeks, 1990; Streichert and Weeks, 1995), and the fates of the interneurons are unknown. The proleg motoneurons exhibit two pupal fates: death or re-specification. All of the PPRs, the APRs in segments A5 and A6, and their target muscles die in response to the prepupal peak of ecdysteroids (Fig. 2; reviewed in Weeks *et al.*, 1996). Recent experiments utilizing cell culture indicate that the ecdysteroids act directly on proleg motoneurons to activate an intrinsic program of cell death

(Streichert and Weeks, 1994; reviewed in Levine and Weeks, 1996). The APRs that survive in segments A3 and A4 grow new dendritic arbors, are respecified for new functions, and die after adult emergence (Fig. 2). In segment A4, the APRs' larval target muscle, APRM, degenerates and the APRs appear to innervate a new abdominal extensor muscle (Weeks and Ernst-Utzschneider, 1989). In segment A3, the larval APRMs persist in reduced form during the pupal stage. After the prolegs degenerate, these APRMs insert on the ventral abdominal wall at a location that is covered externally by the wings of the developing adult moth (see Fig. 2). At pupation, the APRs and APRMs in segment A3 begin to be driven in a rhythmic motor pattern that circulates hemolymph within the lumen of the developing wing (Sandstrom, 1993; Lubischer *et al.*, 1995 and unpub. data). This is a remarkable example of functional respecification: returning to Dethier's analogy, the neuromuscular system of the hands (prolegs) has been respecified to function like a heart! We plan to investigate the ontogeny of synaptic inputs that drive APRs in the new circulatory motor pattern that, intriguingly, begins to be expressed when the APRs' dendrites are maximally regressed. Studies of how the APRs are incorporated into this new circuit will complement our previous work examining how the APRs are phased out of the PWR circuit at the end of larval life.

Short-Term Modification of the PWR Circuit During Learning

Prior to the dismantling of the PWR circuit at pupation, *Manduca* spend about 2 wk in the larval stage. Laboratory-reared *Manduca* spend their larval life in plastic cups containing artificial diet. This habitat does not resemble their natural environment: the stems and foliage of solanaceous plants. When provided with plants, larvae cling with their prolegs and thoracic legs and spend most of their time stationary, either feeding or "resting." In this posture, the PHs typically project parallel to the substrate and are undeflected; stimuli that deflect the PHs include movements of the foliage produced by the wind or the activity of other insects. In a cinematographic analysis of *Manduca* larvae, Reinecke *et al.* (1980) noted that feeding can be interrupted by external disturbances, potentially affecting growth rate. Therefore, it may be advantageous for larvae to disregard routine stimuli that are nonthreatening. This issue has not been addressed in a natural setting but, under more controlled conditions, we have now shown that PWR exhibits the two simplest forms of nonassociative learning: habituation and dishabituation. Habituation is a decrease in the amplitude of a response to a repeated stimulus, whereas dishabituation is an increase in a decremented (habituated) response after presentation of a novel or noxious stimulus (Thompson and Spencer, 1966). These forms of plasticity serve to filter out mundane sensory stimuli while leaving the system sensitive to novel, and thereby potentially significant, stimuli. Below we describe behavioral and electrophysiological experiments that investigate these short-term modifications of PWR function in *Manduca*.

Habituation and dishabituation of PWR behavior

Habituation and dishabituation of the PWR were tested in isolated larval abdomens, which show fewer spontaneous movements and greater stereotypy of the PWR than do intact insects (Weeks and Jacobs, 1987). The stimulus, a brief deflection of a single PH, was delivered by an automated apparatus (Fig. 4A). The response, measured as the evoked force of proleg withdrawal, was measured by an isometric force transducer attached to the proleg tip. Dishabituating stimuli were delivered by pinching the body wall dorsal to the experimental proleg with a pair of forceps.

Figure 4B shows a representative example of habituation and dishabituation of the PWR. The PH was deflected for 500 ms once every 60 s for 25 trials. The evoked force of proleg withdrawal decreased significantly between trials 1 and 20, reflecting habituation of the PWR, and pinching the body wall between trials 20 and 21 caused a significant recovery on trial 21, demonstrat-

ing dishabituation. The PWR exhibits several of the defining characteristics of habituation (Thompson and Spencer, 1966), including spontaneous recovery from habituation following the cessation of stimulation, an effect of interstimulus interval on response decrement, and rehabilitation following dishabituation (Wiel and Weeks, 1992, 1996).

Although all of the neural elements required for the PWR are present in the ganglion of the same segment (Fig. 1B), we did not know whether these neural elements are likewise sufficient to mediate habituation and dishabituation of the reflex. To address this question, the PWR was tested after surgically isolating the ganglion of the experimental segment from the rest of the CNS. In these neurally isolated body segments, PWR habituation was robust but dishabituation was negligible (Wiel and Weeks, 1996; Wiel, 1995). Thus, habituation can be produced within a single body segment, whereas dishabituation involves intersegmental processes.

Neural correlates of PWR habituation

We next sought to identify the locus or loci within the PWR circuit that are responsible for response decrement during habituation. Peripheral mechanisms such as sensory adaptation or muscle fatigue could contribute, as could central changes within the PWR circuit. A semi-intact electrophysiological preparation consisting of an abdominal ganglion and attached proleg tip (Trimmer and Weeks, 1989) was used to test whether central neural changes were involved. To eliminate the possible contribution of sensory adaptation, a train of electrical pulses that mimicked the normal pattern of PH sensory neuron activity during a 500-ms deflection was applied to a PH socket to activate its sensory neuron. The PH sensory neuron was activated in an identical pattern on each stimulus trial. To eliminate the possible contribution of muscle fatigue, the response to each stimulus was measured as the number of action potentials (spikes) evoked in the ipsilateral proleg motor nerve. In response to the same stimulation protocol used in isolated abdomens (20 trials, 60 s interval), the motoneurons exhibited a significant response decrement, representing a central neural correlate of habituation (Wood *et al.*, 1994, and in preparation; Wiel, 1995). Moreover, following a 30-min rest after trial 20, the response recovered spontaneously to prehabituation levels, indicating that the response decrement resulted from repeated stimulation rather than nonspecific factors. Thus, changes in the central neural circuit for the PWR contribute to habituation.

Where in the circuit do these changes occur? The monosynaptic excitatory connections from PH sensory neurons to proleg motoneurons (Figs. 1B, 3A) were likely sites of plasticity for two reasons. First, homosyn-

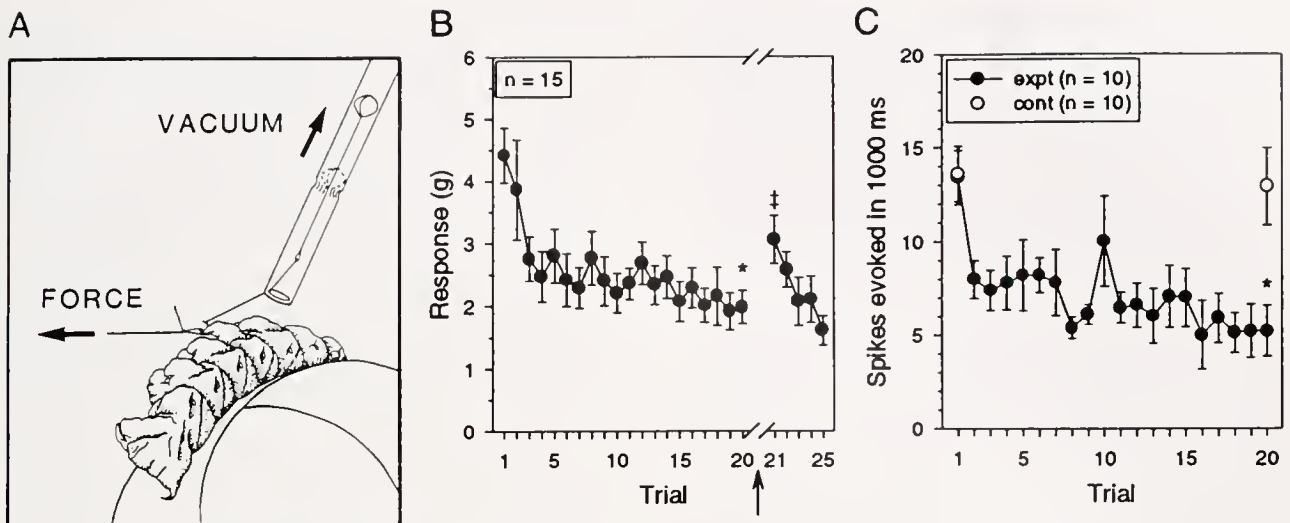


Figure 4. Habituation and dishabituation of the PWR. (A) Behavioral preparation. An isolated abdomen was glued to a curved platform and a single PH was deflected by attachment to a small styrofoam ball that was sucked up a pipette by controlled negative pressure (VACUUM). The force of proleg withdrawal was measured (in grams) by an isometric force transducer (FORCE) attached to the proleg tip. (B) Habituation and dishabituation in isolated abdomens. A PH was deflected for 500 ms at 60-s intervals for 25 trials, and the evoked force of proleg withdrawal was calculated as the difference between the peak force during the PH deflection minus baseline force before the deflection. Thirty seconds after trial 20, the body wall dorsal to the experimental proleg was pinched with forceps (arrow). Significant habituation occurred between trials 1 and 20 (* indicates $P < 0.01$), and significant dishabituation occurred between trials 20 and 21 ($\ddagger$ indicates $P < 0.01$); $n = 15$ abdomens. (C) Decrease in activity evoked in PPR by repeated PH sensory neuron stimulation. The preparation consisted of an isolated proleg tip and the ganglion of the same segment. An intracellular recording was made from the ipsilateral PPR. A train of electrical pulses that mimicked the normal pattern of sensory neuron activity during a 500-ms PH deflection was applied to a PH sensory neuron. Experimental preparations (expt) received 20 trials at a 60-s ISI, whereas control (cont) preparations received 2 trials at a 19-min ISI. The response to each stimulus was measured as the number of spikes evoked over baseline in PPR, during the 1000 ms following stimulus onset. PPR's response decreased significantly between trials 1 and 20 in experimental ($n = 10$; * indicates $P < 0.01$) but not in control ($n = 10$; $P > 0.05$) groups. Values in (B) and (C) are mean $\pm$ SEM. Data from Wiel and Weeks (1992, 1996), Wiel *et al.* (1995, and in preparation) and Wiel (1995).

aptic depression of synaptic transmission from sensory neurons to postsynaptic neurons is a primary mechanism of habituation in several other systems, including the gill- and siphon-withdrawal reflexes of *Aplysia* and the tail flip escape reflex of crayfish (reviewed by Krasne and Glanzman, 1995). Second, previous experiments involving PPR demonstrated that these connections exhibit considerable short-term plasticity. The EPSPs are cholinergic and mediated by nicotinic acetylcholine receptors (nAChRs) on PPR (Weeks and Jacobs, 1987; Trimmer and Weeks, 1989, 1993). There are also two classes of muscarinic cholinergic actions: activation of presynaptic muscarinic acetylcholine receptors (mAChRs) reduces EPSP amplitude (Trimmer and Weeks, 1989), whereas postsynaptic activation of mAChRs on PPR activates an inward cationic current that lowers PPR's spike threshold (Trimmer and Weeks 1989, 1993; Trimmer, 1994). In addition to the muscarinic effects, EPSP amplitude is affected by three types of

activity-dependent plasticity—facilitation, depression, and post-tetanic potentiation—which come into play under different patterns of sensory neuron activity (Weeks and Jacobs, 1987; Trimmer and Weeks, 1991). These phenomena appear to be homosynaptic and caused by alterations in neurotransmitter release from sensory neuron terminals. Two forms of plasticity at the synapses—the activation of presynaptic mAChRs and activity-dependent homosynaptic depression—decrease EPSP amplitude and were thus possible candidates for mediating PWR habituation.

Before examining EPSP amplitudes, we first confirmed that PPR's evoked activity decreased during habituation training. This was suggested by the finding that evoked activity in the proleg motor nerve decreased during habituation training (see above) but, in these whole nerve recordings, PPR's spikes could not be distinguished from those of other motoneurons. Accordingly, we repeated this experiment while recording intracellu-

larly from PPR and measuring its response directly. As shown in Fig. 4C, the number of spikes evoked in PPR decreased significantly over 20 trials delivered at 60-s intervals. Furthermore, in control preparations that received just two stimuli, one at the time of trial 1 and the second at the time of trial 20, the number of spikes evoked in PPR did not change significantly (Fig. 4C). Thus, response decrement resulted from repeated presentation of the stimulus.

Finally, using the same experimental and control protocols, we measured the amplitude of EPSPs evoked in PPR by the stimulated PH sensory neuron before and after training. PPR's membrane potential, spike threshold and input resistance, and the magnitude of paired-pulse facilitation of the EPSP were also measured. Contrary to our expectation, the amplitude of the unitary EPSP evoked in PPR by the PH sensory neuron used for training did not change detectably in either experimental or control preparations (Wiel *et al.*, 1995, and in preparation; Wiel, 1995). Therefore, it appears unlikely that either of the two known decremending forms of plasticity contribute to PWR habituation. Of the other parameters measured, PPR's spike threshold became significantly more hyperpolarized and input resistance significantly increased in both the experimental and control groups (Wiel *et al.*, 1995 and in preparation; Wiel, 1995). Because these values changed in both groups, they cannot account for habituation; furthermore, both of the changes would tend to make PPR more excitable and therefore *less* likely to exhibit a response decrement.

These data suggest that PWR habituation is mediated neither by changes in synaptic transmission from PH sensory neurons to motoneurons nor by changes in the intrinsic electrical properties of the motoneurons. The former finding was surprising, given the range of plastic mechanisms known to be in place at these synapses (see above). The functional role of plasticity at these connections therefore remains elusive. These findings indicate that we must look elsewhere in the circuit for the changes that mediate habituation. One possibility is suggested by studies of habituation of the *Aplysia* and crayfish reflexes mentioned above. In both cases, homosynaptic depression of sensory synapses occurs but, in addition, inhibitory activity in interneurons increases during training and causes a reduction in motor output (Fischer and Carew, 1993; Krasne and Teshiba, 1995). Further experiments will be required to investigate whether inhibition contributes to habituation of the PWR in *Manduca*.

Conclusions

The PWR of *Manduca* has been useful for investigating how a neural circuit can be modified under different circumstances. The ecdysteroid-mediated regression of

proleg motoneuron dendrites and the resultant decrease in sensory input provides a mechanism to explain the progressive weakening and loss of the reflex at pupation. Some of the neuromuscular components are "recycled" as members of the pupal circulatory system. In larvae, the reflex is modified by experience and, despite the known plasticity in synaptic transmission from PH sensory neurons to proleg motoneurons, the properties of these synapses appear to remain stable during habituation. Thus, the specific neural mechanisms that underlie habituation of the PWR elude us at present. On a larger scale, the PWR is only one of many behaviors that involve the prolegs, and the relationship between elements of the PWR circuit and the circuits that produce these other behaviors remains unexplored. The caterpillar abdomen and its "hands" should continue to provide insights into how the nervous system balances constancy with change when generating behavior.

Acknowledgments

Research funding was provided by NIH (R01 NS23208, K04 NS01473 and T32 GM07257), an NSF Presidential Young Investigator award (J.C.W.), and grants from the Whitehall Foundation, M.J. Murdock Trust, Oregon Medical Research Foundation and the U.S. Department of Education. We thank Dr. Devon E. Wiel for providing unpublished data, and Dr. Wiel and Dr. William M. Roberts for comments on the manuscript.

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Dynamic Encoding of Odors With Oscillating Neuronal Assemblies in the Locust Brain

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Abstract. The computational rules followed by the brain to encode complex, multidimensional stimuli such as natural odors are not well understood. In this review, we summarize results obtained in the olfactory system of an insect and present a hypothesis for odor representation in the brain. We propose that individual odors are represented by ensembles of neurons that are distributed both in space (the specific identities of the neurons forming an ensemble) and in time (the time at which each neuron participates in the ensemble response). In addition, we discuss the potential roles that periodic synchronization (oscillations) might play in this complex process.

Introduction

Odors in the natural world are usually complex blends of many volatile compounds. The percept that each natural fragrance evokes in us, however, is usually singular (*e.g.*, a rose, garlic, or a skunk). Our brains, therefore, probably form a unique internal representation of each specific blend, from which individual components (such as amylacetate, for example) must be difficult or impossible to segment. This specific odor representation must, in addition, be stable over time (odor memories are very

long-lasting), and sufficiently inclusive to allow like odors (*e.g.*, roses of distinct varieties) to be “classified” as of the same sort.

A major challenge of neuroscientific research on olfaction is to understand the “computational” rules used by the brain to encode complex stimuli such as odors. Remarkable recent developments in vertebrate molecular biology allow us to understand some important aspects of mapping of odor-signals in the olfactory bulb (Buck and Axel, 1991; Vassar *et al.*, 1994; Sullivan *et al.*, 1995). These results complement physiological and imaging studies of odor processing indicating broad, distributed representation schemes (Cinelli *et al.*, 1995). Other recent fascinating results from studies of molluscan olfaction indicate that the olfactory nervous system of an invertebrate generates oscillations (Gelperin and Tank, 1990; Delaney *et al.*, 1994), a macroscopic functional feature similar to one long described in vertebrates (Adrian, 1942; Freeman, 1978; Satou, 1990). This result, combined with anatomical evidence that olfactory circuits in arthropods, molluscs, and vertebrates are built along very similar architectures, suggests that the computational rules used by olfactory systems may be similar (or conserved) across animal phyla and classes.

In this short review of our work, we summarize the dynamic and distributed scheme according to which we think odors are represented in the first and second olfactory relay stations of an insect brain. We address the issues of combinatorial coding, temporal representation, synchrony, and oscillations. The hypotheses presented here rely on recent electrophysiological, immunocytochemical, and morphological data from our laboratory, presented in Laurent and Naraghi (1994), Laurent and Davidowitz (1994), Leitch and Laurent (1996), and Laurent *et al.* (1996). We apologize to all authors whose

Received 30 November 1995; accepted 19 March 1996.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

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work is not cited and discussed here, and rather send readers to several excellent recent reviews on related and overlapping topics (Cinelli and Kauer, 1992; Freeman, 1992; Gray, 1994; Axel, 1995; Hildebrand, 1995; Hammer and Menzel, 1995; Singer and Gray, 1995).

Odor Representation in the Locust Olfactory System

In this section we review our main electrophysiological findings and derive from them a hypothesis for odor-coding, which might be applied to olfactory systems other than those of insects. When an odor (for example a pine fragrance) reaches the antenna of a locust (*Schistocerca americana*), a subset of the ca. 300 local and 850 projection neurons in the ipsilateral antennal lobe is activated. This subset may comprise 10% to 20% of the total complement of neurons in the antennal lobes. These activated neurons do not, however, all respond at the

same time, or in the same way. We consider here two important and overlapping aspects of these response patterns: first, the slow and odor-specific temporal patterns of activity; second, the synchrony of firing and the oscillations of local field potential (Fig. 1).

Slow, odor-specific response patterns

When an odor is delivered to the antenna, some projection neurons fire for a few hundred milliseconds at the onset of the stimulus, while others fire only after a certain, consistent delay (from 10 to several hundred milliseconds). Other projection neurons yet may fire during 2 (or more) distinct epochs but remain silent in between. In other words, the ensemble of neurons that fire simultaneously (*i.e.*, within any 100-ms period of the ensemble response) changes as time progresses. This progressive change in the ensemble activated by an odor, how-

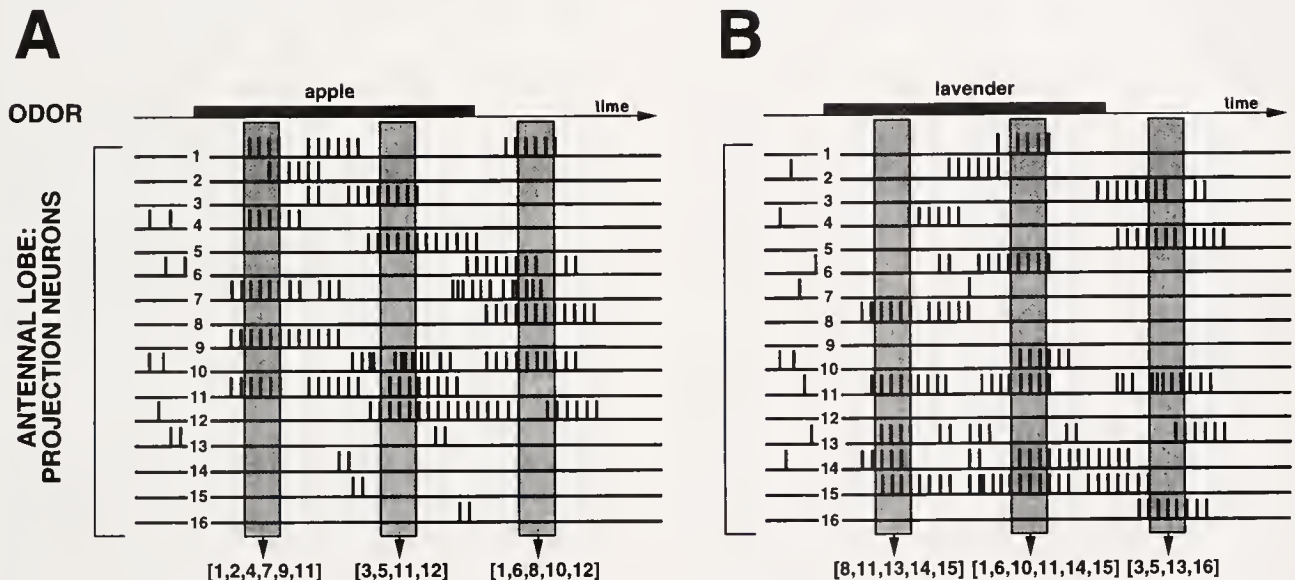


Figure 1. Distributed coding scheme for odors in the locust olfactory system. Each odor evokes spike activity in a subset of projection neurons in the antennal lobe (10%–15% of total complement of projection neurons). All responding neurons do not, however, respond at the same time. In the synthesized response represented in A, for example, neurons 1, 2, 4, 7, 9, and 11 respond together during epoch 1 (first gray window), whereas neurons 3, 5, 10, 11, and 12 respond together during the second highlighted epoch. Hence, the ensemble of neurons whose simultaneous activity represents the stimulus (here apple) changes progressively throughout the response. Superimposed on this distributed, evolving representation are synchronizing events, such that most of the active neurons in any one epoch fire together and periodically (20–30 Hz). The coherent firing of these subsets of neurons causes periodic activation of Kenyon cells in the mushroom body; this activation can be evidenced as local field potential oscillations with the same frequency. Because the membrane potential of individual Kenyon cells oscillates in response to presentation of the appropriate odor, it is hypothesized that the representation of the stimulus is refined in the mushroom body, by “selecting” inputs *only* from those projection neurons that are synchronized. In the example in A, for example, projection neuron 10 is not synchronized to the other active neurons (3, 5, 11, 12) during the second highlighted epoch. We propose, therefore, that the representation of the stimulus at this time is carried by neurons 3, 5, 11, and 12. The distributed representation obeys the same principles for each odor. In B, lavender is represented by a different evolving (overlapping) subset of projection neurons.

ever, is reliable over repeated presentations of the same odor, and is odor-specific. Indeed, presentation of a different odor (*e.g.*, a citrus fragrance) leads to the formation of a different (but partially overlapping) ensemble of activated projection neurons, with its own specific temporal features of activation. It appears, therefore, that a part of the odor representation relies on a specific spatial activity pattern (formed by the "physical" members of the odor-activated ensemble), as well as a specific temporal activity pattern (defined by the order in which the different neurons are recruited). Because both the spatial and temporal aspects of the response are reliable and odor-specific, we hypothesize that both must play a role in the encoding and "internal representation" of the odor signal.

Synchrony and 20–30 Hz oscillations

Intermeshed with the slow temporal activity patterns described above are more subtle aspects of neuronal activity: Antennal lobe neurons indeed often respond to odors not only by producing action potentials during specific time periods around the odor delivery, but also by locking these action potentials to a 20–30 Hz field potential oscillation that can be recorded extracellularly from the ipsilateral mushroom body. This field potential oscillation, which is synchronous over the entire mushroom body, is in fact the result of synchronous firing of a group of antennal lobe projection neurons, activated by an odor.

One first important point is that the seemingly uninterrupted 20–30 Hz field potential oscillations recorded in the mushroom body in response to an odor stimulus are caused by a sequential activation of groups of antennal lobe projection neurons. Indeed, because individual projection neurons generally respond only during a short period of the population response, each successive oscillation cycle is caused by the synchronous firing of a slightly different—and progressively evolving—subset of the activated projection neurons. If one could image the *ca.* 1100 antennal lobe neurons simultaneously and use the oscillation cycle duration (*ca.* 50 ms) as the "frame rate," one would see a specific succession of images created by the unfolding odor-evoked dynamical activity pattern.

A second important feature is that each projection neuron may not participate in the synchronized ensembles during the entirety of its response. Indeed, a projection neuron may produce action potentials during a significant period of the ensemble response but synchronize only a portion (*e.g.*, the first half, or the middle third) of these action potentials to the field potential. In other words, the fact that a projection neuron produces action

potentials during an odor response does not necessarily mean that it participates in the ensemble oscillations during all (or any) of its response. The period during which a projection neuron synchronizes with the field potential oscillation (if it does) is, however, reliable and odor-specific. This indicates that it is not sufficient to consider only the number of action potentials produced by a projection neuron to understand its potential contribution to the ensemble representation. Rather, one must consider the precise timing of each action potential relative to the local field potential, *i.e.*, to the synchronous ensemble. Only a few of the action potentials contained in a response are generally phase-locked to the field potential and thus participate in the coherent activity reflected as oscillations in the mushroom body. The representation of an odor therefore appears to rely on an evolving population of synchronized projection neurons. Each projection neuron generally participates in the synchronized ensemble for a duration shorter than the ensemble oscillations, either because it is silent, or because it is not phase-locked during some of the ensemble response.

A third important feature of these oscillations relates to the phase of firing of individual projection neurons. When a projection neuron synchronizes to the local field potential, each spike occurs at a relatively precise phase (relative to the corresponding cycle of the field potential) that appears, so far, to be independent of the nature and concentration of the odor. Similarly, odor- or concentration-specific phase sequences that would indicate a coding scheme using phases or delays (Von der Malsburg and Schneider, 1986; Hopfield, 1995) have not been observed.

Where are these oscillations (or rather, the synchronization leading to the oscillations) generated? Ablation experiments showed that the calyx of the mushroom body is not necessary for the production of 20–30 Hz oscillatory response patterns in individual local and projection neurons in the antennal lobes. This suggests that the oscillations originate in the antennal lobes. Experiments in progress in which a GABA receptor antagonist is injected in various brain loci indicate that synchrony is abolished when inhibition is blocked in the antennal lobe but not when it is blocked in the mushroom body (MacLeod and Laurent, 1995). These observations are in agreement with ones made with the vertebrate olfactory system, showing that communication between olfactory bulb and piriform cortex is not required for the generation of odor-evoked theta (5–10 Hz) and gamma (30–60 Hz) oscillations in the olfactory bulb (Gray and Skinner, 1988).

To summarize, these data suggest that odors are represented by dynamic ensembles of neurons, and that the

oscillations evoked by odor presentation act, at least in part, as a "carrier wave" for a message distributed both among neurons (spatial representation) and in time (temporal representation). This message is "encoded" in the antennal lobe and sent to the mushroom body, where it may lead to a specific associative pattern representing the memory of this odor. The code is thus combinatorial, in that each odor is represented by a specific ensemble, and each neuron on its own can convey to the experimental observer only a little information about the identity of the stimulus.

Why Oscillations?

Our description of oscillations in the olfactory system is, of course, not the first. Odor-induced oscillations were first described in mammals more than 50 years ago (Adrian, 1942; Gray and Skinner, 1988), and were later observed in fish (see Satou, 1990) and molluscs (Gelperin and Tank, 1990; Delaney *et al.*, 1994). Our findings of oscillations in the locust olfactory brain and more recently in the cockroach and honey-bee brains (Stopfer and Laurent, 1995) add to the notion that processing in olfactory systems may use the same fundamental principles (or algorithms) across most animal phyla and classes (Hildebrand, 1995). But why oscillate at all? Although the existence of oscillations does not, in our minds, constitute the core of the hypothesized encoding scheme described above, it is a conspicuous (one might even sometimes argue distracting) component of it. For this reason, we will speculate here on why olfactory systems might oscillate.

One first and obvious possibility is that oscillations by themselves serve no particular purpose, but rather result simply from the functional architecture of olfactory networks. Oscillations, in this scheme, are only a by-product of processing by circuits built with prominent negative feedback loops (local-projection neuron interconnections). This hypothesis is plausible, given the similarity of microarchitecture of the olfactory circuits in the primary olfactory relays of insects, crustaceans, molluscs, and vertebrates. It is functionally rather uninteresting, however, because it assigns no functional purpose to neuronal synchronization.

A second possibility relates to the issue of learning. Maybe the ultimate goal of this part of the brain is to exploit synchronous activity patterns to form memories of the stimuli, using coincidence-dependent learning rules (*e.g.*, Hebbian learning rule; see for example Bourne and Nicoll, 1993). Indeed, it is important to realize that oscillations are the result of synchronization of large numbers of neurons, but that synchronization *per se* does not require oscillations (Singer and Gray, 1995)!

One might indeed imagine nonperiodic synchronized activity patterns that would lead to nonoscillatory extracellular field potential activity. The problem is that, given the nature of biophysical and physiological systems, it is much easier to generate periodic than nonperiodic synchronous patterns. The existence of stimulus-induced oscillations might, in this context, therefore be a by-product of the "need" to produce synchrony for memory formation. Oscillations would therefore only be "useful" in the sense that they underlie coincident activity, with a "learning purpose." This hypothesis is more tempting (or pleasing), because it starts placing neuronal architecture, activity, and synaptic physiology into a coherent whole, and one that seems ultimately required for olfaction.

A third hypothesis is that setting up oscillations *de facto* creates a new variable—phase—with which to encode stimuli. Indeed, the existence of oscillations defines a periodic "clock" signal, in relation to which the timing of individual action potentials can be measured (Von der Malsburg and Schneider, 1986). Many theoretical arguments have been built that favor such a mechanism for purposes as diverse as segmentation (the separation of stimulus features such as object from background) in vision or hearing (Von der Malsburg and Schneider, 1986) or concentration-independent odor perception in olfaction (Hopfield, 1995). At present, however, the available data do not support these hypotheses, for only occasionally has phase been observed to vary, and to vary reliably, over repeated stimulus presentations (rat hippocampal place cells; O'Keefe and Recce, 1993). In most cases in which oscillations have been described, the phase of individual action potentials relative to the oscillations has been shown to be either remarkably consistent (under certain stimulus conditions) or inconsistently variable (under other stimulus conditions). To caricature these observations, it seems that the phase of spikes relative to the population (average) signal is either a constant (to a degree) or *unpredictably* variable. In other words, no consistent phase signature appears to be suggested by the available data. We therefore do not have any data supporting this third hypothesis at present.

A fourth hypothesis relates to a problem posed by distributed, combinatorial neuronal representations of sensory stimuli. If a stimulus (*e.g.*, an odor) is represented by an ensemble of neurons (rather than by one highly specific neuron, for instance), this distributed representation can be formalized as a vector sum of all the vectors contributed by each participating neuron (Georgopoulos, 1995). In this representation, the space where stimuli lie has as many dimensions as there are component neurons (about 850 here), and each odor can be represented by either a point (if the ensemble is static) or a

trajectory (if the ensemble is dynamic) within that space. The contribution of each neuron (*i.e.*, the length of the vector contributed by each neuron) is therefore measured by the number of spikes it provides to the ensemble. This, naturally, depends on the time over which spikes are counted. If the stimulus, or its representation, is dynamic, one needs a clock signal to update this population vector as the stimulus, and the response to it, both unfold. The oscillations may provide this clock signal, with the following added advantage: We observed that, if a neuron synchronizes to the field potential, it usually spikes only once per oscillation cycle. In other words, the contribution of each vector to the population vector is 0 (no spike or an unsynchronized spike) or 1 (one synchronized spike) at each oscillation cycle. A possible advantage is that the trajectory of the population vector is confined to the edges of an 850-dimension cube instead of occupying an infinitely large space. In this scheme, therefore, the oscillations would define the clock according to which the vector direction is updated, and simultaneously limit the possible length of this vector and of its trajectory. This type of reduction of the "coding space" might be very important in the design of an optimal memory system, whose role is to store new, and recognize old, distributed odor representations.

A fifth plausible hypothesis is that oscillations filter unwanted action potentials. In the case of the insect olfactory system, this would provide the means to build a first population odor representation in the antennal lobe, using a large subset of the available neurons (10%–20% of them, as suggested by the data), and to form a sparser representation, more convenient for memory storage, in the mushroom body, the second olfactory neuropil, where storage may be implemented (Hammer and Menzel, 1995; Davis, 1993). In this scheme, the Kenyon cells of the mushroom body would, using dendritic voltage-gated nonlinearities for example (see Laurent and Naraighi, 1994), associate inputs only from "perfectly" synchronized neurons, eliminating the contribution of ill-timed action potentials—and hence the contribution of the neurons producing these ill-timed action potentials. This method might thus transform, as well as "trim," the odor representation, making it less likely to saturate the memory capacity of the mushroom body and to lead to large overlaps and thus over-generalization.

Conclusions

Most of these hypotheses are of course nonexclusive and compatible with each other. They are also only some of many that space limitations (and limited imagination) prevent us from formulating here. The experimental problems facing us now are formidable. For any one of

these hypotheses to be proven valid, it will have to be tested using a combination of refined physiological, behavioral, and psychophysical techniques. For example, one will want to ask the animal: "Did you smell and recognize odor X?" in conditions where firing synchrony, but not the identity of the firing neurons, is selectively disrupted. Because the tools for such experiments are, unfortunately, still undefined, this remains one of the most challenging tasks for neuroethologists. Our present findings clearly indicate that stimulus-specific temporal patterns of neuronal activity can be evoked in the brain, strongly suggesting that temporal codes (whether they are carried by a coherent oscillation or not) have a role to play in brain function. Whether this role is important, and what it might be, remains to be determined.

Acknowledgments

This work was supported by an NSF–Presidential Faculty Fellow and a McKnight Foundation Scholarship to Gilles Laurent.

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The Role of Attention in Learning About Odorants

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The goal throughout this period was to understand the neural mechanisms mediating chemosensory responses and related behavior. At the same time, it was the behavior that was providing insight to the mechanisms. The behavioral approach was an exciting game of wits [emphasis added]. It proved to be a powerful tactic, and many of its findings were subsequently shown by electrophysiology to be gratifyingly accurate.

—Dethier (1990)

Introduction

Animals must solve several kinds of problems in relation to identification and location of food or mates. One fundamental problem involves recognition of the types of stimuli that help to predict the spatial and temporal occurrence of such resources (Smith, 1993). These stimuli occur in complex, changing backgrounds that might disrupt recognition of critical stimulus elements. Thus the task at hand for the sensory system is to filter out the less relevant background information in order to extract those elements that reliably provide information about the identity and location of the resource (Hopfield, 1991).

To perform this feature-extraction task, nervous systems must be capable of devoting as much of the system's processing capacity as possible to the essential elements of the signal. One approach is illustrated by the pheromone systems of moths and cockroaches (Masson and Mustaparta, 1990). In those animals, specific neural pathways are devoted to processing information about a very restricted set of odorants (Hansson *et al.*, 1991;

Rospars and Hildebrand, 1992). Particular pathways, beginning with the peripheral sensory cells, are typically narrowly tuned to one element of a pheromone mixture or to the appropriate mixture itself. The information content of pheromones is stable over evolutionary time, hence more of the olfactory system's processing capacity can be dedicated (hardwired) to detection of the restricted range of elements in the pheromone.

Feature extraction is a more complex problem for odorants that are not pheromone-like in their information quality; that is, their meaning can change rapidly within the lifetime of any given individual (Smith and Getz, 1994). For example, foraging honey bees learn to associate floral odorants with the nectar and pollen resources that are necessary for colony survival (Menzel, 1990). Thus, as has now been shown in many studies, a floral odorant can act as a conditioned stimulus (CS) to train honey bees by Pavlovian and instrumental/operant conditioning procedures (Bitterman *et al.*, 1983). Odorant information, which together with general distance and direction information is transmitted among individuals during dance recruitment, enables recruits to identify which among several types of flowers in a locale are providing the resource at any given time (Frisch, 1967). The problem is that floral odorants are mostly complex mixtures of dozens to hundreds of component odorants, and the exact nature of the blend can change from flower to flower or within a flower across short periods of time (Pham-Delegue *et al.*, 1989; Knudsen *et al.*, 1993).

In fact this problem of blend recognition is a general one faced by all animals that have even a rudimentary sense of smell. Many biologically relevant odors are mixtures of at least several components, and a number of physiological and molecular-level processes might limit an animal's ability to perceive component odorants or submixtures in a blend (Dionne and Dubin, 1994). Odorants in a blend interact, at least in vertebrates, with potentially thousands of receptor types embedded in sen-

Received 30 November 1995; accepted 3 April 1996.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

sory cell dendrites (Buck and Axel, 1991), and cells that express a particular type of receptor project to the same glomeruli (Vassar *et al.*, 1994). Recent investigations of sensory physiology have shown that odor molecules interact with these receptors to produce several nonadditive effects when odorants occur in a mixture (Ache, 1989, 1991; Atema *et al.*, 1989; Getz and Akers, 1995; Michel and Ache, 1994). Such effects include mixture suppression, which is the decrement of response in one type of cell to a specific odorant due to the presence of additional odorants in a blend. Furthermore, synergistic interactions may recruit new cell populations to respond to the blend even though they show little or no response to the component odorants. Therefore, it is likely that a blend of two or more odorants may take on perceptual properties that are unlike those of any component, and any recognizable quality of the components might be diminished.

The picture presented thus far presents a tremendous challenge to our understanding of learned odor recognition. Olfactory signals are characterized by the production of an input signal that must have an extraordinarily high number of dimensions, and addition or deletion of components in a blend can qualitatively change how the blend is perceived. If, therefore, the perceptual qualities of a blend are so dramatically affected by perturbations in the presence or ratios of components, then how does a bee, or for that matter any animal, recognize the "same" odor despite the high degree of variability across space and time? It may be that different flowers, or even the same flower at different times of day, have slightly different compositions of odorants than those that were experienced at an earlier time. What is needed is a sensory processing system that exhibits "graceful" degradation as the stimulus is altered.

Therefore, an important conceptual problem that faces us in our understanding of the olfactory system is this: How do animals generalize from one experience with a learned odorant to the next experience even though the two presentations may differ? The problem of generalization has played a central role in learning research (Kalish, 1969; Shepard, 1987). Indeed no two presentations of any CS are alike even under controlled laboratory conditions (Kalish, 1969). If an animal were to focus on identification of only the exact CS used for training, it might risk making an inappropriate response on a later presentation. That is, in the extreme case a bee might revisit only the same flower it just depleted of nectar (Smith, 1993). It is easy to understand how that could be fatal to an animal in the short or the long term, especially when such learning helps to predict an imminent encounter with a predator (Hollis, 1984).

One means of dedicating computational resources to particular elements of a complex signal—that is, ensur-

ing that animals generalize to the most relevant aspects of a signal—can be loosely categorized under the general phenomenon of "attention" (Logan, 1992; Holland and Gallagher, 1995). This term subsumes many kinds of processing systems that have at least one fundamental feature in common—the processing capacity can be flexibly dedicated and rededicated to different elements as the information value of those elements rapidly changes. The concept of attention can involve differing degrees of complexity and flexibility in species with different capacities inherent in their central nervous systems. But in all cases it helps animals to filter and process the more relevant elements of a signal that is composed of a complex and changing mixture of elements. The ability to attend to specific elements of a signal while learning other elements performs essentially the same function as the pheromone system described above: it allows animals to attend to the informationally more relevant signal elements while filtering out less relevant ones. This kind of attention system is most relevant for signals that have rapidly changing information content, such that processing capacity cannot be dedicated developmentally as it is in the pheromone systems.

In the spirit of the above quote, it is the point of this contribution that behavioral studies indicate that mechanisms exist by which animals can "extract" component information from odors made from mixtures of two or more components. One such behavioral paradigm, termed "blocking," is highlighted. Paradigms such as blocking have been employed to study how animals learn about mixtures of stimuli from different sensory modalities. But paradigms can also be effectively used to study processing within modalities. Using information from these behavioral studies, we can now make testable predictions about the nature of signal processing, beginning with sensory receptors and proceeding inward, that can explain how animals solve the variance problem with odors. Many of these studies highlight what amounts to an ability to flexibly devote neural computational resources to processing one or a few signal components. That is, they describe processes of attention, which may vary in complexity across taxa. But first, it will be useful to review a more commonly accepted model of olfactory processing and generalization.

The Problem of Stimulus Generalization

As defined above, the essential problem is to determine how animals generalize from one odor to another so as to appropriately respond to an odor that is slightly different from the one to which they were trained. Stimulus generalization can arise and vary among individuals as a result of a variety of factors (Kalish, 1969). A typical means for studying generalization would be to train sub-

jects to a single CS, for example, a tone, and then test the same subjects by varying some dimension of the CS, such as frequency or intensity. In such a protocol generalization, which is defined as a response to a stimulus that represents an alteration in some property of the CS, is frequently presumed to occur because the dimension along which the CS is varied (*e.g.*, frequency, wavelength, intensity) is represented in the peripheral and central nervous systems. The dimensions represented in the nervous system can be set up developmentally—that is, by the array of receptors expressed across sensory cells (Buck and Axel, 1991)—as well as by associative or cognitive mechanisms that form new dimensions as a result of experience (Shepard, 1987).

Generalization in the olfactory system has been studied in honey bee workers trained to respond to a pure odorant CS (Smith and Menzel, 1989; Smith, 1991). The same subjects are subsequently tested with odorants possessing the same oxygen moiety (*e.g.*, ketone, aldehyde, or alcohol) but different carbon chain lengths, or with odorants having the same chain length but different oxygen moieties. For most odorant types fairly smooth generalization gradients can be established. That is, subjects respond very strongly to the CS and slightly less so to an odorant similar in structure to the CS but varying along some dimension. The response becomes progressively weaker as the structure of the molecule is changed even more along one or two dimensions. Similar data, albeit using electrophysiological responses of mitral cells, have been obtained recently in the rabbit (Mori, 1995).

Generalization from a mixture to one of its submixtures or to one of the components can occur *via* the same type of mechanism as for pure odorants if relevant dimensions can be defined in the nervous system. For example, peripheral sensory interactions between odorants in a binary mixture ($A + X$) could give rise to a neural representation for the mixture that is different from those for either component (A or X alone). That is, the neural elements activated by $A + X$ could be qualitatively different from what would be expected given a simple summation of the elements activated by A and X alone (Pearce, 1994). Indeed such “configural” elements from a mixture could be the ones to which relevant associations with an unconditioned stimulus are made. The greater the difference between the sensory/neural representation for the mixture and those of the two components, the weaker the generalization from the mixture to either component (Fig. 1A).

This type of model was proposed by Hull (1952; see review in Pearce, 1987) to explain “overshadowing” in the same terms that were used to account for generalization within a defined dimension to a single CS. A typical overshadowing experiment would compare principally two groups (Table 1A). One group (OVERSHADOW) is

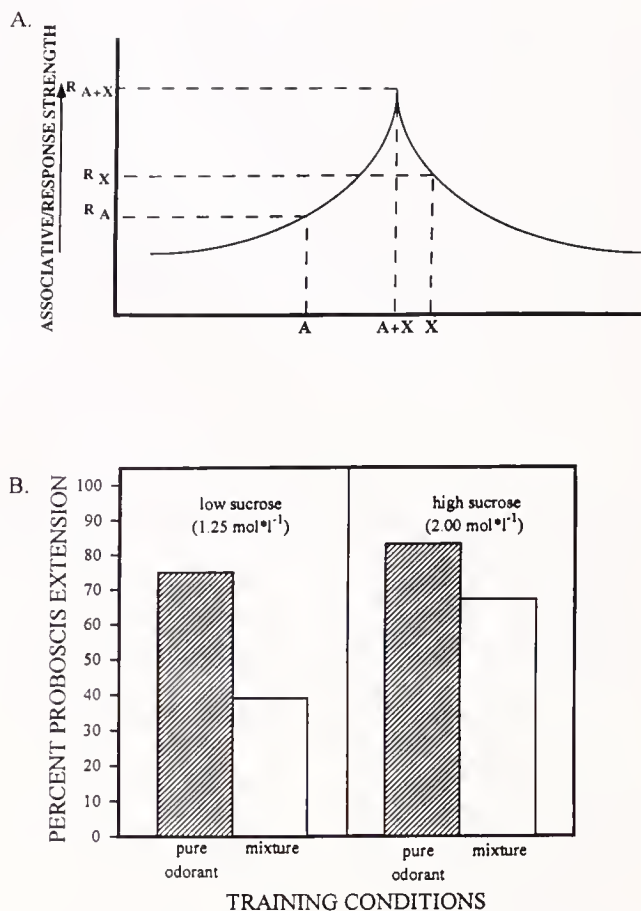


Figure 1. Overshadowing due to afferent interaction at the sensory receptors/cells. (A) Schematic diagram of a model in which afferent interaction between A and X occurs to give rise to a sensory input that is unique to the $A + X$ mixture. The mixture-unique pattern defines a point in a sensory input pattern at which a strong associative strength exists with the neural representation of a reinforcer. The response strength to either A or X would be a function of the similarity of the neural representations of those elements to that of the mixture. The “sensory” space is shown here as a single dimension for simplicity; but it is most likely to be multidimensional in character (Shepard, 1987). (B) Overshadowing of odorant X conditioned in a mixture relative to when conditioned as a pure odorant. Four groups of honey bee workers were conditioned *via* standard protocols for proboscis-extension conditioning (Bitterman *et al.*, 1983; Menzel, 1990). All subjects were exposed to a single acquisition trial during which odor was paired with sucrose reinforcement. Two groups were conditioned to a 50:50 mixture of two odorants ($A + X$), and the remaining two were conditioned to a pure odorant (X) at the same concentration as when it was presented in the mixture to the former group. (1-Hexanol and geraniol were counterbalanced as A and X .) All groups were subsequently tested with a single presentation of the same pure odorant (X) that was not reinforced (*i.e.*, an extinction trial was performed). Subjects were scored as having responded to odorant X or not; thus the figure shows the response probabilities in the respective groups. This experiment was performed with a low concentration of sucrose in two groups and a substantially higher concentration in the remaining two groups. The response in both mixture-trained groups ($n = 18$ subjects in both cases) is lower than in the respective pure-odorant-trained groups ($n = 24$). However, the difference is significant only when a lower level of reinforcement is used (LOW: $\chi^2 = 5.58$, $P < 0.05$; HIGH: $\chi^2 = 0.69$, NS).

Table 1

Summary of experimental designs used in overshadowing (A) and blocking (B) experiments

	Pretraining	Blocking	Test
A. PURE ODORANT:	$X \rightarrow \text{sucrose}$	— ¹	X
OVERSHADOW:	$A + X \rightarrow \text{sucrose}$	— ¹	X
B. BLOCK:	$A \rightarrow \text{sucrose}$	$A + X \rightarrow \text{sucrose}$	X
NOVEL:	$N \rightarrow \text{sucrose}$	$A + X \rightarrow \text{sucrose}$	X
OVERSHADOW:	— ¹	$A + X \rightarrow \text{sucrose}$	X

¹ For an overshadowing experiment there is no training during the second phase of the experiment. The two OVERSHADOW groups are equivalent even though the phase during which no conditioning takes place is shown to be different between A and B.

conditioned to a mixture of stimuli, which for our purposes would be two odorants ($A + X$). The response to one component (e.g., X) is then tested, and one frequently finds that the response is diminished relative to when the mixture is tested. A second group (PURE ODORANT) is conditioned to and subsequently tested under identical conditions with X . Overshadowing occurs if the response to X is lower in the group trained to the mixture than in the group trained to the pure odorant. Indeed overshadowing occurs in the honey bee under specific conditions of reinforcement (Fig. 1B).

A recent study of cross-adaptation in the honey bee has demonstrated that the overshadowing data can be accounted for by peripheral sensory interaction (Bhagavan and Smith, in prep.). That is, when the sensory cells are adapted to one odorant, the response to the remaining odorant also shows adaptation. Thus there is significant interaction among odorants as they compete for limiting substrates involved in signaling in the antennal system. Although several mechanisms can account for cross-adaptation, these data provide circumstantial evidence to support the interpretation that overshadowing can also be accounted for by afferent interaction in primary signal transduction processes. This interaction would be capable of giving rise to unique qualities of $A + X$ and would produce overshadowing *via* a process represented in Figure 1A. Note, though, that overshadowing can also be accounted for by other processes; for example, by a decrement in the concentration of odorant that occurs between training of $A + X$ and testing of X (the same decrement does not occur in the PURE ODORANT group; Table 1A). Furthermore, the same attention-based models used to account for blocking (see below) can also account for overshadowing (Rescorla and Holland, 1982). Further experimentation will be required to resolve these interpretations of overshadowing in the olfactory system.

Situations in which subjects have little or no prior ex-

perience with stimuli used for conditioning to mixtures (as, for example, in an overshadowing protocol) may provide valuable, albeit conservative, information regarding how the olfactory system is constrained by afferent interaction caused by specific stimulus configurations. The complexity of the peripheral coding system now emerging from molecular studies (Buck and Axel, 1991) would certainly lead us to expect that mixture representations arising from peripheral sensory-level interactions would occur and account for a number of the behavioral effects of mixtures with varying numbers of components (Laska and Hudson, 1993). But do these kinds of studies indicate absolute limits to an animal's ability to discriminate mixture components? The answer to that question is probably "no."

Blocking as One Solution to the Generalization Problem

Several studies in recent years have shown that prior experience with an odorant can enhance an animal's ability to detect that odorant in a mixture. Experiments involving selective attention in humans have demonstrated that the ability of subjects to perceive a target component can be improved at least in a limited way (Rabin and Cain, 1989; Laing and Glemarec, 1992). In these protocols, human subjects are instructed to attend to one component and then discriminate that odorant from an adulterated version of itself. Under controlled conditions such familiarity can improve the ability of subjects to detect elements of mixtures.

The learning phenomenon called "blocking" is one of several means of studying different kinds of attention in nonhuman animals (Rescorla and Holland, 1982; Rescorla, 1988; Holland and Gallagher, 1995). Blocking occurs when a subject is conditioned to one stimulus (X) in the presence of another (A) that has been previously paired with the same reinforcer used for subsequent conditioning of the $A + X$ compound. The response to X after such conditioning is typically lower than when the subject receives $A + X$ pairing (i.e., an overshadowing treatment) with no prior conditioning of A . This learning paradigm is important because it shows that proper spatial and temporal pairing of X with a reinforcer is not a sufficient condition to increase learning performance to X . Furthermore, it allows an animal to generalize from a learned stimulus (e.g., A) to a condition in which A occurs embedded in a different blend or background (i.e., $A + X$) that might degrade the animal's ability to recognize the presence of A .

In both *Limax* (Sahley *et al.*, 1981) and honey bees (Smith and Cobey, 1994), blocking has been demonstrated among odorants in mixtures. That is, prior learning about one odorant will block learning about a new odorant when the latter is subsequently provided in a bi-

nary mixture with the first. Both studies incorporated several control procedures (Table 1B). In the honey bee, subjects in group BLOCK were pretrained with several conditioning trials during which odorant *A* was forward-paired with a sucrose *US*. One control group of subjects (NOVEL) received equivalent pretraining to a different, novel odorant in order to equilibrate exposure in this group to odorant and to the *US*. A second control group (OVERSHADOW) received no pretraining. All groups received equivalent training to the *A* + *X* mixture in the subsequent blocking phase. Finally, all groups were tested for their responses to odorant *X*.

The results demonstrate that the response to *X* is significantly lower in group BLOCK than it is in groups NOVEL or OVERSHADOW (Fig. 2). That is, acquisition to odorant *X* is blocked or at least significantly retarded when it occurs in a context in which the reinforcer is already adequately predicted. Note that groups did not differ in their exposure to odorant *X*; all of them received equivalent exposure to *X* in a mixture with *A*. Also note that the level of response in the OVERSHADOW group already represents a decrement in responding (see Fig. 1B) and that the blocking protocol *increases* the level of this effect; that is, the response to *X* is lower in group BLOCK than it is in group OVERSHADOW.

Several control procedures have shown that simple exposure to the *US* and to odorant *A* is not sufficient to produce blocking. When *A* is either backward-paired or explicitly unpaired with the *US* in the pretraining phase (that is, using pairing conditions that would not be expected to build up excitatory associative strength to *A* but would equilibrate exposure to it across groups), blocking did not occur (Sahley *et al.*, 1981; Smith and Cobey, 1994). Therefore, blocking results specifically from forward- (associative-) pairing of odorant *A* with the *US* and not from a nonspecific process such as cross-adaptation of sensory cells responsive to *X* by pre-exposure to *A*. Finally, it should be noted that the decrement in concentration that occurs in an overshadowing experiment, and which could potentially account for overshadowing, occurs equally across all groups in a blocking experiment. Therefore, even if that decrement contributes to overshadowing, it cannot account for differential response levels in a blocking experiment.

It is impossible for the generalization decrement model of Hull (1952) to account for the blocking effect in general and particularly for its specificity to the forward-pairing condition (Pearce, 1987). Why should prior forward-pairing *increase* an overshadowing effect that is, according to that model, due to peripheral interactions? Furthermore, sensory adaptation to odorant *A* during pretraining could affect the subjects' responses in the blocking protocol in different ways depending on the level of interaction in the antennal system. Because such

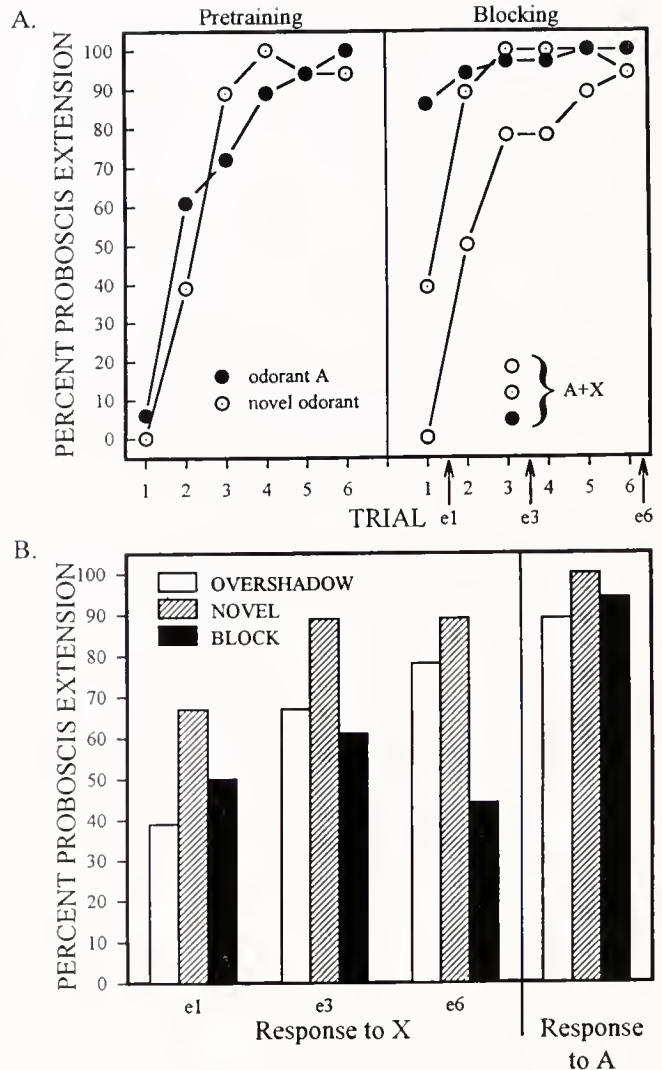


Figure 2. Summary of acquisition and extinction testing in a blocking experiment. (A) Subjects in groups NOVEL and BLOCK were conditioned to a novel odorant or to odorant *A*, respectively, as in Figure 1B, except six forward-pairing acquisition trials were used in the pretraining phase. All groups received six trials with the *A* + *X* mixture in the blocking phase. Geraniol and 1-hexanol were counterbalanced as *A* and *X*, and 2-octanone was used as the novel pretraining odorant. Eighteen subjects were selected and randomly assigned to each group. In order to assess responses to odorant *X*, all subjects received extinction trials with odorant *X* after the first, third (e3), and sixth (e6) trials in the blocking phase. In addition, after all extinction testing was complete subjects received a final extinction trial with odorant *A* to determine whether differences in responding to *X* might be due to a more general mechanism (*e.g.*, low motivational states) than to blocking. (1.25 mol·l⁻¹ sucrose was used for reinforcement throughout the experiment.) (B) The emergence of a blocking effect throughout the course of the blocking phase. Response probabilities to odorant *X* in groups OVERSHADOW, NOVEL, and BLOCK are shown at e1, e3, and e6. The response probability of group BLOCK is significantly lower than those of groups NOVEL and OVERSHADOW at e6 ($\chi^2 = 2.118$, $P < 0.01$) but not at e1 ($\chi^2 = 2.82$, NS) or at e3 ($\chi^2 = 4.28$, NS). One-trial blocking—that is, a significant difference among groups at e1—occurs under other conditions of reinforcement (Smith, in prep.). Groups do not differ in response probabilities to odorant *A* ($\chi^2 = 2.12$, NS).

a nonassociative effect could not be detected (Smith and Cobey, 1994), other mechanisms that give rise to blocking should be investigated. Indeed several behavioral models have been invoked to explain the blocking effect: some involve changing levels of attention to the CS or the US, whereas others are based on recall failure (Rescorla and Holland, 1982; Spear *et al.*, 1990). These models still need to be tested in more detailed behavioral analyses, particularly in regard to the emergence of the blocking effect after a single conditioning trial with the mixture (Fig. 2).

Clearly there are several means that animals might employ to extract and thereby detect components of complex olfactory signals. Thus the point made here is not that blocking is the only tactic available for analyzing mixtures. Specific adaptation of sensory receptors for one mixture element can enhance the ability to detect the remaining elements (Cain and Polak, 1992). Laing *et al.* (1994) have also shown that humans are capable to some extent of distinguishing between fast and slowly processed signal elements. In other words, mixtures can be analyzed perceptually according to the temporal qualities of the components. This latter kind of processing may be particularly useful when the elements of a signal show temporal fluctuations that are uncorrelated to background odors (Hopfield, 1991). The point that must be addressed is how these rules for processing odorant mixtures, particularly the ones that involve attention-like, flexible processing like blocking, are implemented in the CNS.

Implications for Synaptic Plasticity in the Olfactory Lobes

Although odorant blocking may serve the same function as the pheromone system, the neural mechanisms underlying the two systems must be very different. The blocking system would have to involve a greater degree of, or at least different kinds of, synaptic plasticity, which might be expected even in the more peripheral levels of olfactory processing (*e.g.*, the olfactory/antennal lobes). Thus a comparison of the pheromone and blocking systems from the behavioral to neural and molecular levels should provide insight into the multiple means through which the nervous system can solve the same problem inherent in finding resources.

Can we speculate as to where this synaptic plasticity might occur such that it gives rise to blocking? As already mentioned, because of its specific, associative nature, blocking seems unlikely to arise *via* known sensory mechanisms in the antennal system (Smith and Cobey, 1994). For example, if blocking had not been specific to the forward-pairing condition, it could have been explained by cross-adaptation of *X* sensory cells by pre-

exposure to *A*. But in the absence of that result, why would forward-pairing of *A* with the US, but not the other means of pairing the same two stimuli, decrement the response of sensory cells activated by odorant *X* when it is mixed with *A* in the blocking phase? If cross-adaptation could explain olfactory blocking, why would pre-training with a novel odorant not produce blocking?

Therefore, the next level of sensory processing at which synaptic plasticity might occur and give rise to the associative, forward-pairing effects of blocking would be in the antennal lobe (AL; Flanagan and Mercer, 1989; Linster and Masson, 1996), which is structurally analogous to the olfactory bulb of vertebrates (Shepherd, 1991). As sensory axons enter the AL, they project to glomeruli, within which all known synaptic interactions with interneurons in the brain take place (Homborg *et al.*, 1989). It is here that projection neurons, the anatomical invertebrate equivalent of mitral cells, receive input from sensory neurons, although the exact mechanism of this input is still unknown in invertebrates. In addition to these two types of cells, local interneurons are also involved in input/output relationships within glomeruli. One such class of local interneurons, which has arborizations limited to the AL, is GABAergic and presumably capable of spreading inhibition either globally within the AL or throughout a limited subregion (Flanagan and Mercer, 1989; Homborg *et al.*, 1989). Such inhibition is crucial for the ability of projection neurons to track, for example, temporal patterns in pheromone stimuli (Christensen and Hildebrand, 1988).

One other type of interneuron, which has recently been physiologically and immunocytochemically characterized, plays a modulatory role in several brain neuropils (Hammer, 1993). In recordings made from arborizations of this neuron in the mushroom bodies, which are downstream from the ALs, Hammer (1993) has shown that this neuron responds very little to odorant stimulation prior to association of odorant with a US, but that the odorant response increases after forward-pairing. These data indicate that this neuron, named VUMmx1, is capable of representing the US in neural models of associative conditioning, and thus it is an essential element in an associative network in the brain.

The VUM neuron also arborizes extensively within most if not all of the glomeruli within the AL (Hammer, 1993). If this neuron is capable of providing a neural representation of the US and thus mediating synaptic plasticity within the mushroom bodies, then it might be involved in similar associative modification within the AL. Although the argument presented here is only circumstantial, precedents from studies of vertebrates indicate that synaptic plasticity can alter the neural representation of an odorant in the accessory olfactory bulb (Brennan *et al.*, 1990) and in the olfactory bulb of rats (Leon,

1992; Sullivan and Wilson, 1991; Wilson and Sullivan, 1991) and sheep (Kendrick, 1995).

How might this synaptic plasticity alter the neural representation of odorant *A* during the pretraining phase? Voltage-sensitive-dye recordings from the salamander olfactory bulb show specific spatial and temporal patterns of glomeruli activation when an odorant is presented (Cinelli *et al.*, 1995). It could be that these patterns change as a result of association of the odorant with reinforcement. The activation of glomeruli specific to an odorant at a given concentration could be enhanced; new glomeruli could be recruited; or some aspect of the temporal pattern such as speed or oscillation (see Laurent, 1996; Laurent and Davidowitz, 1994) could be changed. Even if the pattern of excitation did not change, the pattern of local inhibitory transmission might be altered as a result of changes in synaptic drive to inhibitory interneurons.

This is only a partial list of what might be affected by associative conditioning, but it indicates some changes we might search for to test the hypothesis proposed here. The result of any such change could be that patterns of lateral inhibitory transmission would be substantially altered as a result of conditioning. If this inhibition is strengthened, then it might be capable of suppressing a representation evoked for odorant *X* when it is subsequently added to pretrained odorant *A*. This kind of mechanism would give rise to a blocking effect because, if activation of elements corresponding to odorant *X* is prevented by the activation of those corresponding to *A*, then *X* might not be as capable of entering into an association with the reinforcer.

In summary, the mechanisms underlying olfactory blocking may be a means through which the peripheral olfactory system, which includes the AL, deals with the high dimensionality of the input signal from the peripheral sensory system. By acting like a filter of sorts, blocking could cause the representation of a mixture to be biased, or *conditioned*, to be much more like that of a few elements that are, for one reason or another, the most important elements of the signal. A mixture of *A* + *X* might be perceived to be much more *A*-like after pretraining with the latter element. This reduction in dimensionality of the input might ease the processing load placed on neuropils, such as the mushroom bodies, that are downstream from the AL and must also integrate information from other sensory modalities into the olfactory information coming from the AL. The end result would be stronger generalization from a conditioned odorant to a mixture that contains that odorant.

Conclusions

The type of attention system that gives rise to blocking is bound to be different from more cognitive models of

attention studied in selective-attention protocols. Nevertheless, this blocking protocol provides access to studies of flexible processing of stimulus mixtures in all animals. In the end we should not be surprised if blocking cannot always recover information about an odor as it is embedded in different backgrounds. But the effect may simply bias the mixture representation to make whatever task is at hand (*e.g.*, stimulus generalization) easier for a later stage of processing. And the results can, as I have attempted to show, provide testable hypotheses about how blocking can be implemented in the CNS. At this point the model proposed above needs to be more thoroughly vetted mathematically and experimentally. But modification in the AL must also be investigated as explanations of the blocking effect are sought in other brain regions downstream from the AL. This model I have proposed may seem to some like too much speculation about behavioral data. But, as Dethier wrote, the study of behavior is, after all, an *exciting game of wits!*

Acknowledgments

Support for this work was provided from NIMH award #1 R29 MH47984 to BHS.

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Temporal Shifts in Foraging Strategy: Introduction

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I was pleased to be invited to participate in the symposium honoring Vince Dethier. In many ways Vince was a model scientist. Throughout his career he worked on several different questions. In each case, he devised systematic empirical attacks on substantial problems. The essence of his general approach was presented in his book "*To Know a Fly*." This book was written in an amusing style and was intended for first- and second-year undergraduate students and the general public. But despite its nontechnical, humorous approach (or, perhaps, because of it) I have found that this book is extremely effective in introducing scientific method to students in lower level undergraduate courses such as introductory psychology. This, I think, is a testament to the clarity of Vince's thought (and writing).

At a more personal level, I was on the faculty of the University of Massachusetts in Amherst for many years. During that time Vince joined the University and helped us in many ways, serving as director of the Neuroscience and Behavior Program, and adding visibility and distinction to the program. More importantly, Vince was a valued colleague for many of us in the program.

Vince Dethier's work on the control of feeding behavior in blowflies examined the classical issue that, to a psychologist, defines motivation: explaining why the same organism behaves differently at different times as a function of changes in the organism's internal state. In classical motivational research, this question focuses upon such sources of variation as hunger and thirst.

The two papers that follow expand on this basic theme of Dethier's work. In each case, the applicability of De-

thier's work to a current, important problem in behavioral ecology and ethology is clear. In the first paper, Dave Stephens (1996) considers the puzzle of the effects of short delays in delivery on the value of food reward. From a functional point of view, it is very difficult to understand why an animal should prefer a small, immediate reward to a large, but slightly delayed, reward. Stephens explores the application of the type of mechanism Dethier discovered to the behavioral ecology of feeding in this problem. Although the exploration is only a preliminary one, it demonstrates how fruitful this approach may be.

The second paper, by Lucia Jacobs (1996), takes as its theme the question of changes in behavior and internal state over time. In recent years, it has become apparent that such changes are important, not only over relatively short time scales, but over longer ones. Some of the more interesting of these cases involve food storage and recovery. Many animals deal with seasonal changes in the availability of food by storing food when it is abundant and recovering the stored reserve later, when food is otherwise scarce. In her paper, Jacobs examines some of the changes in brain structure and behavior associated with food storing. Although it deals with a relatively long time span, from season to season, the theme is clearly similar to that underlying Dethier's work on feeding in blowflies. Taken together, these papers demonstrate the applicability of Dethier's ideas to current problems in understanding the behavior of animals.

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This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

On the Relationship Between the Feedback Control of Digestion and the Temporal Pattern of Food Intake

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The Time Scales of Foraging Behavior

One of the simplest experiments in animal psychology is a straightforward evaluation of the effects of time and amount on animal feeding preferences. At intervals an animal is offered a choice between two options: one leads to a small amount of food quickly, while the other leads to a much larger amount of food after a longer delay. In these situations, vertebrates abhor delay (I don't know of any studies with invertebrates). One can think of this pattern of preference as a situation in which increasing time devalues amount, and by fitting classic "decay" functions to this kind of data we can estimate the power of this effect. It is astonishing. For feeding bluejays (studied in my laboratory, Stephens *et al.*, 1995) the "perceived value" of amount decays by roughly 10% per second (see Kagel *et al.*, 1986 for cogent discussion of the quantitative behavior of this decay phenomenon).

This temporal "discounting" of food, together with several similar phenomena studied in the operant laboratory, paints a picture in which consequences *now* are the primary determinants of animal feeding preferences. It is as if animals care only about the very short term (the next few seconds). Of course, a moment's reflection tells us that this can't be the whole story. Many features of animal feeding behavior seem to be organized (from an evolutionary point of view) for longer term goals. Clark's nutcrackers (*Nucifraga columbiana*) diligently harvest and cache piñon seeds in the fall that they consume in the spring (Kamil and Balda, 1991). Migratory birds increase their food intake and put on weight several weeks ahead of actually beginning to migrate (Carpenter *et al.*,

1983). Indeed, the widespread and important phenomenon of learning is best viewed (Stephens, 1991) as a scheme that capitalizes on environmental regularities that occur on a timescale that is certainly longer than seconds.

In short, animal feeding behavior seems to be sensitive to a tangle of differing timescales: in some instances only the very short-term consequences of behavior seem important; in others the longer term consequences are clearly important.

I had this problem in mind when, coincidentally, I was invited to participate in this symposium in honor of Vince Dethier. I thought of Dethier's work on the regulation of ingestion and digestion *via* feedback mechanisms (Dethier, 1976) and began to wonder whether we might be able to understand some of these "timescale" effects by looking inside the animal. Indeed, the mathematical technique of singular perturbation, which is often used to study feedback systems (Murray, 1989; Logan, 1987), represents a tantalizing possibility: in singular perturbation one finds a separate characterization of the short- and long-timescale behavior of a system, and typically these characterizations are quite different. Could this be a clue as to why animals seem to have different feeding preferences at short and long timescales? In the next few pages I present a preliminary attempt to answer this question.

Behavioral ecologists are surprised when they see animals preferring a small amount of immediately delivered food over larger amounts that are delayed (Stephens and Krebs, 1986). One way to think about this problem is shown in Figure 1, in which rate of ingestion is plotted as a function of time. To keep things simple, I think of the time course of eating as long gaps where nothing is eaten punctuated by jumps in ingestion rates. Notice that even if we hold the average rate of intake constant, there are many possible patterns ranging from small, frequent

Received 30 November 1995; accepted 15 April 1996.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

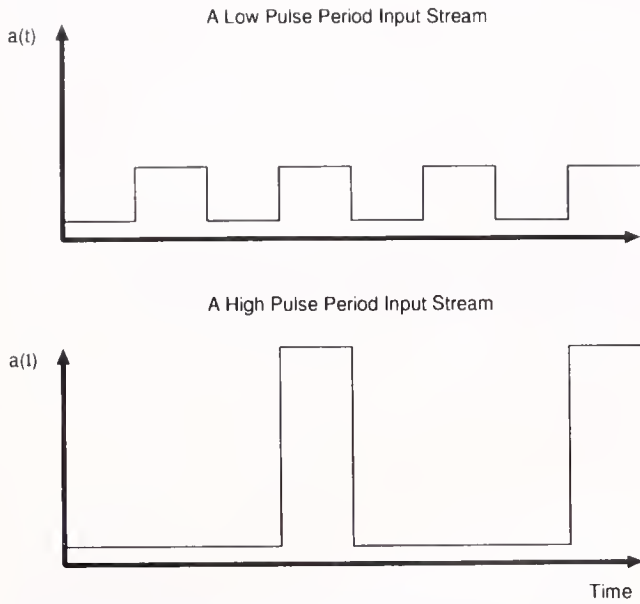


Figure 1: Two input streams give similar overall intake rates but differ in their temporal organization.

blips in intake rate to infrequent, large ones. For a constant average rate, these possibilities differ in the periodicity of bouts of ingestion. In the following model I will ask whether a simple feedback-regulated digestive system does better or worse (i.e., extracts nutrient more or less efficiently) as I vary the “pulse period” but hold the average intake rate constant.

Figure 2 shows the simplified feedback system that I will analyze. To keep the terminology concrete, I will call the first compartment the “crop” and the second the “gut,” and I will suppose that the crop is simply a storage organ in which no digestion takes place, and that all digestion occurs in the gut. I appreciate, of course, that this is a vast oversimplification of any real biological system, even of Dethier’s *Phormia* after which it is modeled. I suppose that inputs to the crop are governed by the square wave type of process mentioned above, and I will denote this input process as $a(t)$. The volume of matter in the crop at any instant in time is V_1 , and the volume in the gut is V_2 . Releases from the crop to the gut are controlled by a feedback law; specifically, matter is released at a rate proportional to the product of (a) the difference between the actual gut volume and a “set point” V_0 and (b) the actual volume of the crop. I suppose that there is an upper limit on the amount of matter the crop can hold $V_{\max}$ (so if the crop volume reaches $V_{\max}$ ingestion cannot continue). Finally, I suppose that effluent leaves the gut at constant rate r (there are a number of reasons to view this assumption with suspicion, but we withhold judgment for the moment). This leads to two differential equations for the crop-gut system:

$$\frac{dV_1}{dt} = \begin{cases} a(t) - g(V_0 - V_2)V_1 & V_1 < V_{\max} \\ -g(V_0 - V_2)V_1 & V_1 \geq V_{\max} \end{cases}$$

$$\frac{dV_2}{dt} = g(V_0 - V_2)V_1 - r$$

We also need two score-keeping equations to keep track of nutrient extraction (the benefits derived from the input stream). First, we consider the absolute amount of nutrient in the gut at time t , say $A(t)$. If C_j is the concentration of nutrient in the material flowing from the gut, then the rate at which nutrient enters the gut is $C_j g(V_0 - V_2)V_1$. At any instant the concentration of nutrient in the gut is $A(t)/V_2(t)$, and so nutrient is lost with the effluent at rate $r A(t)/V_2(t)$. The other process that removes nutrient from the gut is nutrient extraction. The process of nutrient extraction may be quite complex, and it may be qualitatively different for different nutrients. I model extraction as a “Fick’s Law” style absorption process: that is, nutrient is “captured” by diffusing across the gut wall while some homeostatic process maintains the concentration of nutrient on the outside of the gut wall (i.e., changes in the concentration gradient are due only to changes in the concentration within the gut). This means that nutrient leaves the gut *via* extraction at a rate proportional to the wetted surface area of the gut (S) and the concentration the nutrient in the gut, aSA/V_2 . Now, if we view the gut as a rigid container, a cylinder with fixed radius, then the wetted surface area will be proportional to V_2 , say $S = bV_2$. This relationship can be complicated by elasticity in the gut wall and by allowing more complex gut geometries. This linear relationship is the simplest one that captured the simple idea that wetted surface area increases with gut volume. Putting this all together, I write an equation for the change in amount of nutrient in the gut:

$$\frac{dA}{dt} = C_j g(V_0 - V_2)V_1 - \frac{A}{V_2} r - abV_2 \frac{A}{V_2}$$

$$\frac{dA}{dt} = C_j g(V_0 - V_2)V_1 - \frac{A}{V_2} r - kA$$

where in the second equation I have cancelled V_2 and combined the two constants a and b into a single constant $k = ab$. I remark that if the gut is not well-modeled as a rigid container (say that it is more elastic than rigid) then this volume cancelation will not be valid. To keep track of the benefit (i.e., nutrient) extracted over time, say $B(t)$, we would integrate the simple differential equation $dB/dt = kA$.

As a first attempt to understand this system, I analyzed it numerically; that is, I choose parameter values more-or-less arbitrarily and simply integrated the differential equations using well-understood numerical techniques (I used a commercial package and 4th order Runge-

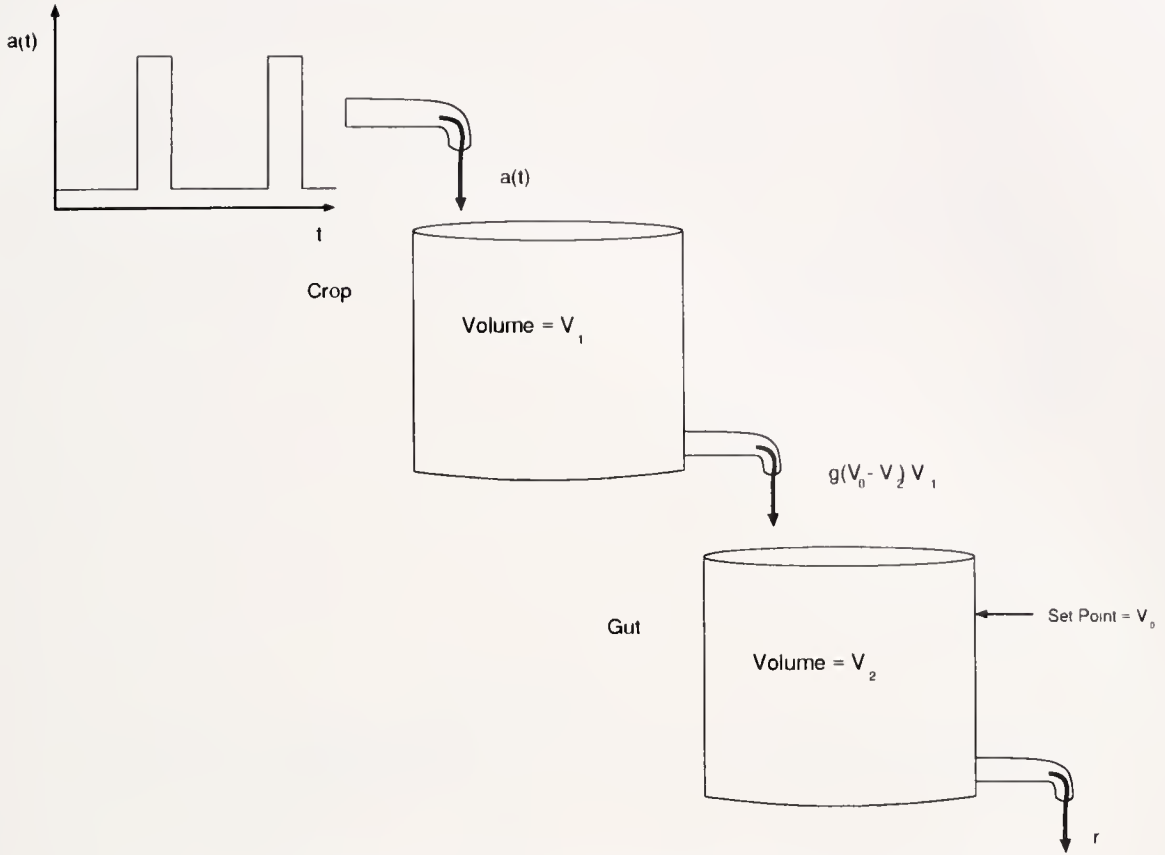


Figure 2: A familiar mathematical caricature of our crop/gut system in which both the crop and gut are modeled as leaky buckets with a feedback control law governing the output from the crop to the gut.

Kutta integration). I tried a range of input functions $a(t)$, all of which were “square waves” that provided the same average input rate but varied in “pulse period.” The relationship that my analysis revealed between nutrient assimilation rate and pulse period is plotted in Figure 3. This is the relationship we would expect if digestive constraints play a part in animal preferences for immediacy: for example, if more even flows of food yield a higher assimilation rate than uneven flows with the same average intake rate, then it follows that it might sometimes make economic sense to prefer an even flow with a low rate of intake over an uneven flow with higher rate of intake. Of course, these numerical results don’t establish this effect as a universal result, they simply suggest that it can happen in some instances. To understand its generality we need to attack the model analytically.

Analysis

A first step in analyzing this system is dimensional analysis (Stephens and Dunbar, 1993), a technique in which one considers the units of each of the model’s variables and parameters and rearranges them into a new, smaller set of parameters and variables that have no units

(i.e., they are dimensionless, or “pure,” numbers). This is important for two reasons. First, it is always easier to work with a smaller number of terms. Second, we need dimensionless numbers to meaningfully evaluate approximate solutions, because we want our estimate of the magnitudes of errors to be independent of the system of measurement used by the experimenters. One way to approach dimensional analysis to pick “characteristic quantities” against which to measure different types of units in the model. In the present model we have volumes (meters cubed), time (seconds), and amounts of nutrient (moles). If we measure volumes in units of $V_{\max}$ and time in units of $1/gV_{\max}$ (a measure of the time required to drain a full crop), and nutrient quantities by $C/V_{\max}$ (the amount of nutrient in a full crop), then we can rewrite our system as

$$\begin{aligned}\frac{d\hat{V}_1}{d\hat{t}} &= \begin{cases} -(\hat{V}_0 - \hat{V}_2)\hat{V}_1 & \hat{V}_1 \geq 1 \\ \hat{a}(\hat{t}) - (\hat{V}_0 - \hat{V}_2)\hat{V}_1 & \hat{V}_1 < 1 \end{cases} \\ \frac{d\hat{V}_2}{d\hat{t}} &= (\hat{V}_0 - \hat{V}_2)\hat{V}_1 - \hat{r} \\ \frac{d\hat{A}}{d\hat{t}} &= (\hat{V}_0 - \hat{V}_2)\hat{V}_1 - \frac{\hat{r}}{\hat{V}_2}\hat{A} - \hat{k}\hat{A}\end{aligned}$$

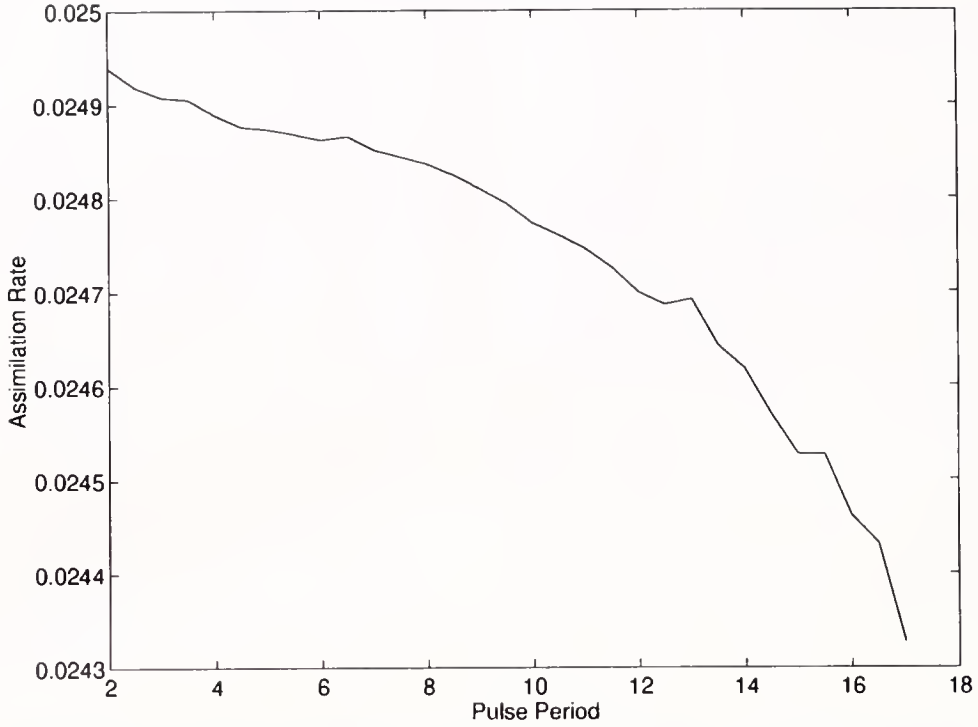


Figure 3: Results of a preliminary numerical analysis of the crop/gut system showing that nutrient assimilation rate declines with increasing pulse period, at least for some parameter values. Recall that the average rate of intake is held constant as the pulse period is increased.

$$\frac{d\hat{B}}{d\hat{t}} = \hat{A}\hat{k}$$

where “hat” symbols denote that fact that the terms have been rescaled. In the remainder of this paper, I shall assume that all terms have been appropriately rescaled, and so the hat symbols will not be used.

The phase plane

As mentioned above, the two volume equations completely determine the system’s dynamical behavior, whereas the two remaining equations are simply mechanisms for score keeping. I focus, therefore, on trying to understand the volume equations. To begin, I consider the collection of crop (V_1) and gut (V_2) volumes where dV_2/dt equals zero (these points represents the so-called V_2 null cline along which gut volume is not changing). If we plot this collection of points

$$V_2 = V_0 - \frac{r}{V_1}$$

in $V_1 - V_2$ space, we have a hyperbolic curve that approaches $V_2 = -\infty$ as V_1 approaches zero, and asymptotically approaches V_0 as V_1 becomes large. Null clines help one understand dynamic systems because we know that the system can only be moving in one dimension

on the null cline (we know that $dV_2/dt = 0$ here, so any movement on this curve must be parallel to the crop volume V_1 axis). To decide whether the system is moving up or down in crop volume, we need to consider the crop volume equation, dV_1/dt .

Since the input stream is a square wave, the crop volume equation takes two forms: (1) a “crop filling” form

$$\frac{dV_1}{dt} = h - (V_0 - V_2)V_1$$

where h is the height of the square wave, or (2) a “crop emptying” form

$$\frac{dV_1}{dt} = -(V_0 - V_2)V_1$$

Of course, along the V_2 null cline, the term $-(V_0 - V_2)V_1 = -r$, so that during emptying phases the crop volumes must be going down $dV_1/dt = -r$ along the null cline; and, assuming $h > r$, crop volume must be increasing $dV_1/dt = h - r$ during filling phases.

Next considering the null clines for crop volume V_1 , we see that for the “filling” equation there is a null-cline at

$$V_2 = V_0 - h/V_1$$

This is exactly same form as the gut volume null cline,

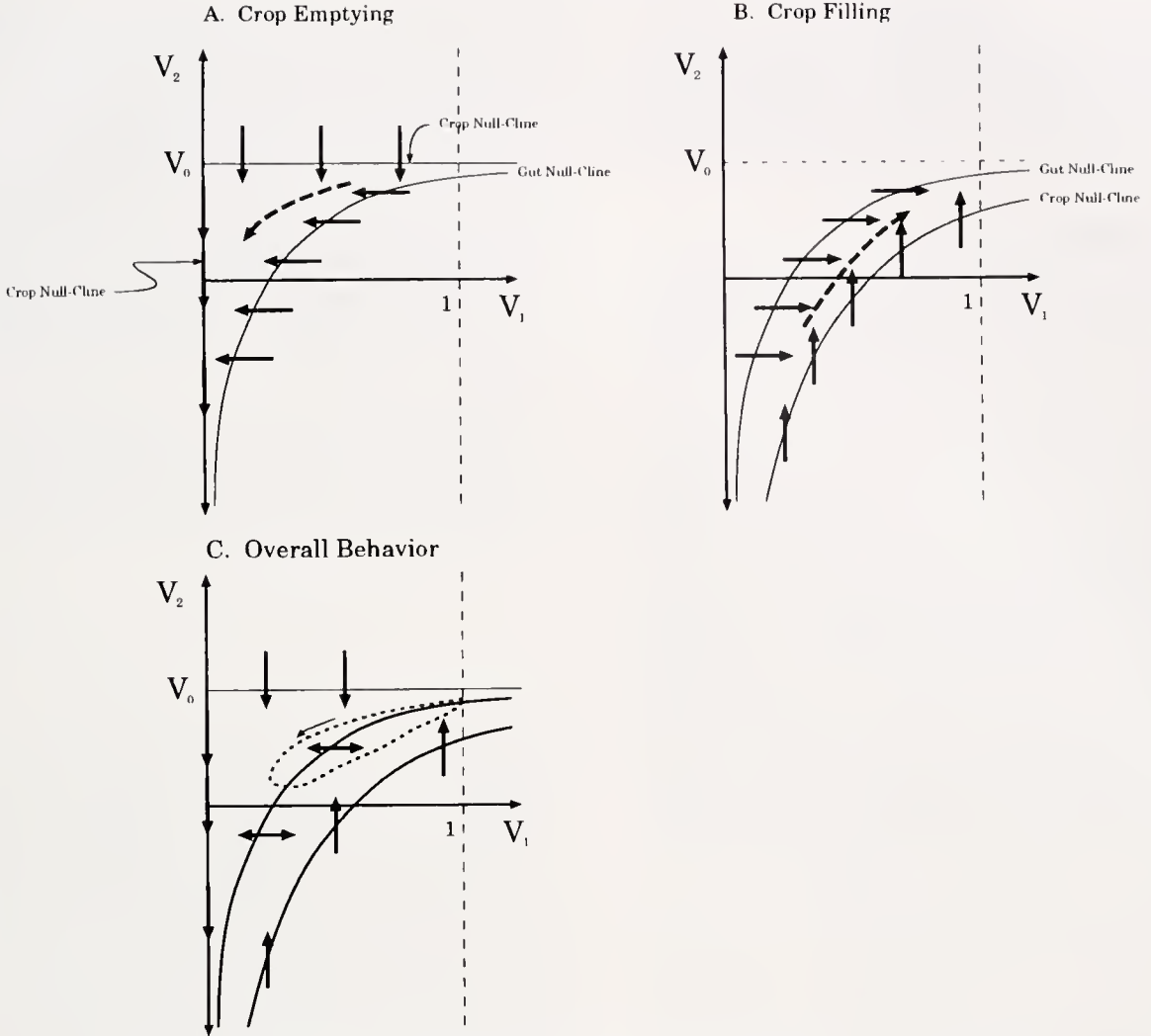


Figure 4: (A) Null-cline analysis of the system when the crop is emptying. The overall is a decrease in both crop and gut volumes. (B) Analysis when the crop is filling. The trajectories are confined between the two null clines, while crop and gut volumes move jointly upward toward the maximum crop volume (which we have taken to be one). (C) Because our system requires that the average intake rate exceed the outflow rate r , a limit cycle will eventually be reached in which an emptying system stays slightly above the gut-volume null cline, whereas a filling system changes direction and climbs back to the maximum volume while staying slight below the gut-volume null cline.

except that h takes the place of r . Since $h > r$ by assumption, this null cline is crudely parallel and below the gut volume null cline. Moreover, since the outflow term $(V_0 - V_2)V_1 = h$ along this null cline, gut volume must be increasing along this null cline, (i.e., $dV_2/dt = h - r > 0$).

For the emptying equation there are two null clines:

$$V_2 = V_0$$

$$V_1 = 0$$

In both cases, of course, the outflow term $(V_0 - V_2)V_1 = 0$, so the gut volume can only be decreasing $dV_2/dt = -r$. These facts are put together in the “emptying” and

“filling” panels of Figure 4. We see that when the system is emptying, the joint (V_1, V_2) trajectory will be attracted to the region between the null clines and then will skirt the gut-volume null cline from above as the crop and gut volumes jointly decline. Similarly, when the system is filling, the trajectory will skirt the gut-volume null cline from below as the volumes jointly increase. I note that our system is a plausible model only when the overall rate of intake exceeds the fixed outflow rate r (simply because an empty gut cannot release material at constant rate r). With this assumption, it follows that at some point, after many cycles of filling and emptying, the crop volume will be at its maximum value. Once this happens

we would expect the system to follow a limit-cycle qualitatively like that shown in Figure 4C: declining from the maximum crop volume while staying slightly above the gut-volume null cline, and then increasing to the maximum crop volume while staying slightly below the gut-volume null cline.

Pseudo-equilibria

The qualitative observation that the trajectory skirts the gut-volume null cline suggests a major simplification of the model; the assumption that $dV_2/dt = 0$. Biologically, this is the claim that the gut volume equilibrates quickly to relatively slowly occurring changes in the crop volume and input stream. Notice that to claim that the gut volume is at equilibrium is not the same as claiming that the gut volume is constant; we are simply supposing that the system's trajectories slide up and down the gut-volume null cline (like a bead on a wire) rather than circulating around the gut-volume null cline as we argued above. The idea of pseudo-equilibrium may be familiar to students of enzyme kinetics; it is part of the traditional derivation of the Michealis-Menten rate law (Murray, 1974). (In addition, one can often build upon a pseudo-equilibrium analysis to construct the "two timescale solutions" that typify the singular perturbation technique.)

Assuming that $dV_2/dt = 0$ changes our system to

$$\frac{dV_1}{dt} = \begin{cases} -r & V_1 \geq 1 \\ a(t) - r & V_1 < 1 \end{cases}$$

$$V_2 = V_0 - \frac{r}{V_1}$$

$$\frac{dA}{dt} = r - \frac{r}{V_0 - \frac{r}{V_1}} A - kA$$

$$\frac{dB}{dt} = Ak$$

Now at any instant, the rate of change of crop volume is either $-r$ in an emptying phase or $h - r$ in a filling phase, so we expect that the volume of crop is either (a) emptying linearly at rate r , (b) full and unchanging, or (c) filling linearly at rate $h - r$. Our earlier numerical analyses confirm that the crop-volume function is approximately piecewise linear. Now consider two square wave input functions that give the same overall input rate R , one with a long period (P_1) and a large peak input rate (h_1), and the other with a short period (P_2) and small peak input rate (h_2) such that

$$R = \frac{wh_1}{P_1} = \frac{wh_2}{P_2}$$

$P_1 > P_2$ and $h_1 > h_2$. In addition, I assume that the aver-

age input rate R exceeds the outflow rate r , as discussed above. Intuitively, it is easy to see that the input stream with the smaller pulse period will, in the long run, produce both a higher mean crop volume and a smaller variance in crop volume. This is essentially a truncation effect: because $R > r$, the system eventually reaches a point at which the crop is full $V_1 = 1$ (in our rescaled units). Starting with a full crop, the long pulse-period system will drain for $(P_1 - w)$ time units at rate r , reaching a minimum crop volume of $1 - (P_1 - w)r$; similarly, the short pulse-period system will drain for the shorter period $P_2 - w$ at the same rate, reaching the larger minimum of $1 - (P_2 - w)r$.

Now, imagine for the moment an experiment in which we hold the crop volume fixed. In this case the equation for the amount of nutrient in the gut (which, in turn, determines the nutrient extraction rate) is strongly attracted to the equilibrium amount:

$$A^* = \frac{r}{k + \frac{r}{V_0 - \frac{r}{V_1}}}$$

Using calculus, it is straightforward to show that this equilibrium amount of nutrient increases with increasing crop volume (the first derivative is always positive) at a decreasing rate (the second derivative is negative for all biologically plausible parameter combinations). This means that increasing the crop volume by one unit has a big effect on the extraction rate if the crop volume is low, but a one-unit increase in crop volume has only a small effect if it is already high. This downward curvature means that varying crop volumes are a bad thing; this stems from a basic mathematical result called Jensen's inequality (see Stephens and Krebs, 1986, chapter 7, for an elementary discussion).

This conclusion suggests that even intake streams provide two economic advantages over uneven ones. First, they allow the system to maintain a higher mean extraction rate because of the truncation effect discussed above. Second, lower variance in crop volume translates into higher extraction rates, because the amount of nutrient in the gut increases with crop volume at a decreasing rate. In light of these kinds of considerations one sees predigestion storage organs like the crop of *Phormia* as devices to attenuate oscillations in intake. So perhaps behavioral ecologists should not be so surprised that animals have strong preferences about the temporal organization of food intake.

Discussion

I have considered a simple caricature of the relationship between food intake and digestion. Indeed, I have

only sketched an analysis of this system. Despite its defects, my analysis shows a possible link between digestive regulation and an important outstanding problem in feeding ecology—animal preferences for immediate food reward. Many complications await further analysis: What if more realistic reaction kinetics are used? What if there is feedback control of the evacuation rate r ? What if nutrients are stored after digestion instead of (as well as) before? There have been many mathematical models of digestion: models of optimal passage rate; the effect of gut length and morphology on digestive efficiency, etc. (Yang, 1993; Penry and Jumars, 1986; Penry and Jumars, 1987; Sibly, 1981). Moreover, many studies have quantified the feedback relations involved in “gastric emptying” (McHugh and Moran, 1979; Hainsworth, 1989). Rather than draw explicitly from this literature, I have built an elementary model crudely patterned after the *Phormia* crop-foregut-midgut system studied by Dethier (1976).

Behavioral ecologists have typically tried to explain animal preferences for immediacy by looking for economic forces outside the animal. The most frequently offered explanation is that in choosing immediate food reward animals are anticipating interruptions (say by a predator or a competitor) that might prevent them from actually collecting delayed food (Kagel *et al.*, 1986; Barkan and Withiam, 1989; McNamara and Houston, 1987). My “explanation” differs from this because it looks inside the animal and asks whether preferences for immediacy can be viewed as an attempt to manage the inputs to a regulated digestive system. Both kinds of explanation could operate simultaneously, of course. One would like to compare the magnitudes of predicted effects in the two types of models and, if possible, create experimental situations in which these effects could be assessed and compared.

As a behavioral ecologist interested in feeding behavior, it is difficult to leaf through the pages of *The Hungry Fly* (Dethier, 1976) without a sense of shame. Behavioral ecologists have, by and large, simply passed up the opportunity to incorporate mechanistic details, like those revealed in the Dethier’s work, into their models. I have tried to offer a simple example of how one might combine Dethier-ian mechanism with the behavioral ecology of feeding, but behavioral ecologists have yet to capitalize on the enormous richness of mechanistic detail.

Acknowledgments

I am grateful for the helpful comments of Peter Bednekof and Tom Getty, and for the continued financial support of the National Science Foundation (IBN-8958228 and IBN-9507668).

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The Economy of Winter: Phenotypic Plasticity in Behavior and Brain Structure

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Abstract. Mobile animals must learn the spatial distributions of resources. The cost of foraging increases dramatically for temperate-zone animals during the winter. Two strategies may be used to balance the energetic budget: reducing costs of foraging and reducing need to forage. Both strategies are correlated with changes in brain structure, specifically in the hippocampus, a forebrain structure used by birds and mammals to map spatial distributions of resources. Small mammals that reduce their need to forage, through hibernation or reduction in body size, show a specific reduction in the structure and size of the hippocampus. The costs of foraging can be also decreased by migration to better foraging conditions or by food-storing, both of which decrease the temporal heterogeneity of food resources. Both of these latter strategies are associated with increased hippocampal structure; for food-storing birds, this increase is a seasonal phenomenon. Thus not only behavior, but also learning ability and even brain structures in adult animals, may be phenotypically plastic in response to the changing demands of the environment.

Introduction

To every thing there is a season, and a time to every purpose under the heaven . . .

—*Ecclesiastes, 3:1*

Behavior and neural structure evolve in response to changing environments, not static ones. When interactions between the environment and the genotype result

in a variety of phenotypes, this is called 'phenotypic plasticity' or "the ability of a single genotype to produce more than one alternative form of morphology, physiological state, and/or behavior in response to environmental conditions." (West-Eberhard, 1989). The phenotype includes "all aspects of an organism other than the genotype, from the enzyme products of the genes to learned behaviors and the effects of disease." (West-Eberhard, 1989). Thus questions of plasticity in behavior and neural structure, usually addressed by the discipline of cognitive neuroscience, or the neural basis of cognitive abilities, may be seen as an example of phenotypic plasticity, and fit into the larger framework of evolutionary processes.

Behavior is perhaps the most plastic of phenotypic traits, and as such has long been seen as a unique agent of evolutionary change (Wcislo, 1989). The neural bases of behavior, in contrast, are usually considered to be a constraint on the range of behaviors, tethering them to the information-processing capacity of a species' predetermined brain size and structure. The idea that both behavior and brain can change in response to environmental challenge is relatively new. It was first recognized in the extraordinary ability of forebrain nuclei in canaries to add new neurons in advance of the annual breeding season. This dramatic example of adult neurogenesis in response to photoperiod revolutionized our perception of adult brain plasticity (Nottebohm, 1981). Yet it can also be seen simply as phenotypic plasticity: a change in brain phenotype in response to changes in the environment. Environmental cues trigger a change in the brain structure, which is correlated with an increase in behavioral plasticity, *i.e.*, the ability to produce nuptial advertisement song. The production of song in turn creates more changes in the environment, as females arrive, attracted by the song, and as their arrival initiates the breeding process. The production of offspring, and the

Received 19 December 1995; accepted 18 March 1996.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

increase in individual fitness, is of course the driving force behind this remarkable phenotypic plasticity in behavior and the brain, which is subject to sexual selection (Searcy and Andersson, 1986; Jacobs, 1996).

Thus our understanding of phenotypic plasticity must include not only plastic behavior, such as learning, but must ask whether its neural basis is plastic as well. The question I would like to address in this review is whether such adaptation in the songbird is extraordinary, or whether it is simply our first example of the phenotypic plasticity of cognitive abilities and their neural basis. If the latter, then where should we search for other examples? Clearly we must first identify what animals learn about their environments, *i.e.* their 'cognitive niche' (Jacobs, 1995), and second, the conditions under which the cognitive niche changes.

It is a basic tenet of behavioral ecology that mobile animals must track the spatial and temporal distribution of critical resources, and that such spatiotemporal distributions underlie and predict adaptive patterns of behavior, such as mating systems (Emlen and Oring, 1977). Thus an animal's ability to learn about space is critical to its adaptive response to a changing environment, and spatial learning should evolve in response to the predictability of spatial patterns of resources. Theoretical models of the evolution of learning ability predict that learning evolves in response to intermediate levels of environmental predictability (Stephens, 1991). At the two extremes, the environment is either perfectly predictable or completely unpredictable, so there is no advantage to tracking and learning spatial distributions. But if the environment is predictably unpredictable, then those individuals who can learn to recognize the changes and predict the distributions will clearly enjoy a fitness advantage over their less perspicacious competitors (Stephens, 1991).

The Economy of Winter

For animals living in seasonal environments, the changing of the season is a highly reliable predictor of a change in resource distribution. Shortening daylengths are an unmistakable predictor of a decrease in temperature and a change in the abundance and distribution of food. Thus foraging behavior, and the brain structures that support it, might be expected to show equally predictable changes in response to winter. How might such changes be organized? A simple answer is that there is a reduced energy budget for the winter economy and that to balance this budget, an animal has two options: it can decrease its costs of foraging or it can decrease its requirement for foraging.

Foraging costs could be decreased by adaptive changes in behavior, such as migration to better foraging areas or storage of food, whereas basic requirements can be altered only by changing the metabolic costs of daily activity. Reproduction is expensive; metabolic costs can be largely reduced by postponing reproduction or ceasing to reproduce. In addition, an animal can decrease basic metabolic costs through hibernation or torpor, through reduction in the absolute size of its physical structure, such as its body or brain size, or through a combination of both.

What would be the cognitive consequences of such winter strategies? One might expect to find changes in the behaviors needed to track spatial distributions. In birds and mammals, a large part of this function is mediated by the hippocampus, or hippocampal formation. This is classically defined in mammals as the Ammon's horn and the dentate gyrus; in birds the relevant brain regions are also known as dorsomedial cortex, and include the hippocampus and the area parahippocampalis (Figs. 1 and 2). The hippocampal formation (hereafter referred to as the hippocampus) is an important fore-brain structure in birds and mammals. Damage to this structure has a variety of effects; however, one of the most consistent and important side effects is loss of the ability to learn new spatial relationships among known landmarks (O'Keefe and Nadel, 1978). For example, laboratory rats with hippocampal damage can no longer create shortcuts through a complex spatial environment; every new path must be laboriously discovered rather than extracted from the rat's memory of the landscape. Invertebrate species, which do not have a hippocampus, cannot make such mental shortcuts (Dyer, 1991), which is the definition of a cognitive map (Tolman, 1948). Thus the hippocampus, in birds and mammals, is required to create cognitive maps, or flexible mental representations of spatial arrays (Bingman *et al.*, 1990; O'Keefe and Nadel, 1978). Further, as might be predicted from this function, the size of the hippocampus within and between species is directly correlated with space use. Its relative volume (volume relative to the size of the whole brain) varies among species with different foraging tactics, and between males and females of the same species when they differ in their space use. For example, a larger home range or a specialized foraging ability, such as scatter hoarding, predicts a relatively larger hippocampus. Closely related species or members of the opposite sex that face lesser spatial demands have relatively smaller hippocampi (Sherry *et al.*, 1992, 1993; Jacobs, 1995).

Because winter is a time of changed spatial distributions of resources critical to individual fitness, such as food and receptive breeding partners, one might predict that a brain structure involved in learning about these

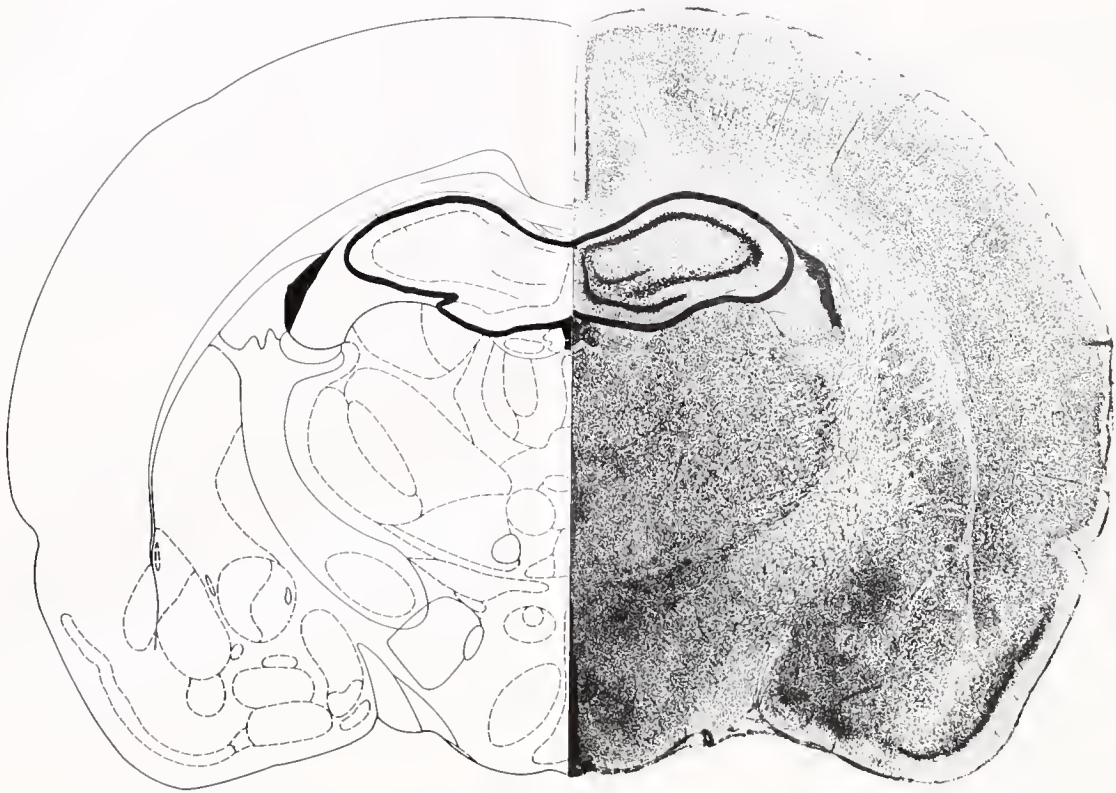


Figure 1. Diagram of a coronal cross-section of the hippocampal formation in the laboratory rat. The right side of the figure shows the Nissl-stained brain section; the left side of the figure shows the boundaries of brain regions. The hippocampus (Ammon's horn and dentate gyrus) are outlined. Adapted from Paxinos and Watson (1986).

distributions would also change with the seasons. It should again be noted that the hippocampus is also involved in nonspatial brain functions, such as the ability to discriminate odors (Eichenbaum *et al.*, 1992) and is not involved in some spatial abilities, such as the ability to remember the location of an object (Cave and Squire, 1991). However, structures may have more than one function, and we would not expect that the size of a structure could be predicted from each of disparate functions, but only from its primary function. In rodents and birds, the evidence strongly favors the hypothesis that the primary function of the hippocampus is to create mental representations of space (Nadel, 1991). Thus I will limit the following discussion to the question of how spatial behavior and the hippocampus may adapt to winter in temperate-zone birds and mammals, according to the two types of winter strategies: reduction of foraging need and reduction of foraging cost.

Reducing foraging requirements

An important strategy in reducing the need to forage is to decrease body size and hence decrease absolute en-

ergetic needs. Many small mammals such as the meadow vole (*Microtus pennsylvanicus*) show reductions in body weight of up to 20% in winter or in short daylengths (Dark and Zucker, 1985).

Another winter strategy employed by small mammals in the temperate zone is to undergo hibernation, dropping their body temperature to a few degrees Celsius (Carey, 1993). This considerable savings in metabolic expense might be expected to have dramatic effects in the brain. Because a hibernating animal lives in an environment that requires no processing of new spatial information, one might expect concurrent decreases in the allocation of brain space used for such processing. Although this conjecture may seem far-fetched, such changes have been demonstrated in hibernating Siberian ground squirrels. The dendritic structure of hippocampal neurons changes dramatically between the state of torpor, an awakened state (such as 2 h after provoked arousal) and a spontaneously active state (between torpor bouts, after spontaneous arousal). In the middle of hibernation, dendrites are significantly shorter and less branched, and they have fewer dendritic spines than dendrites in squirrels who become spontaneously active between hiberna-

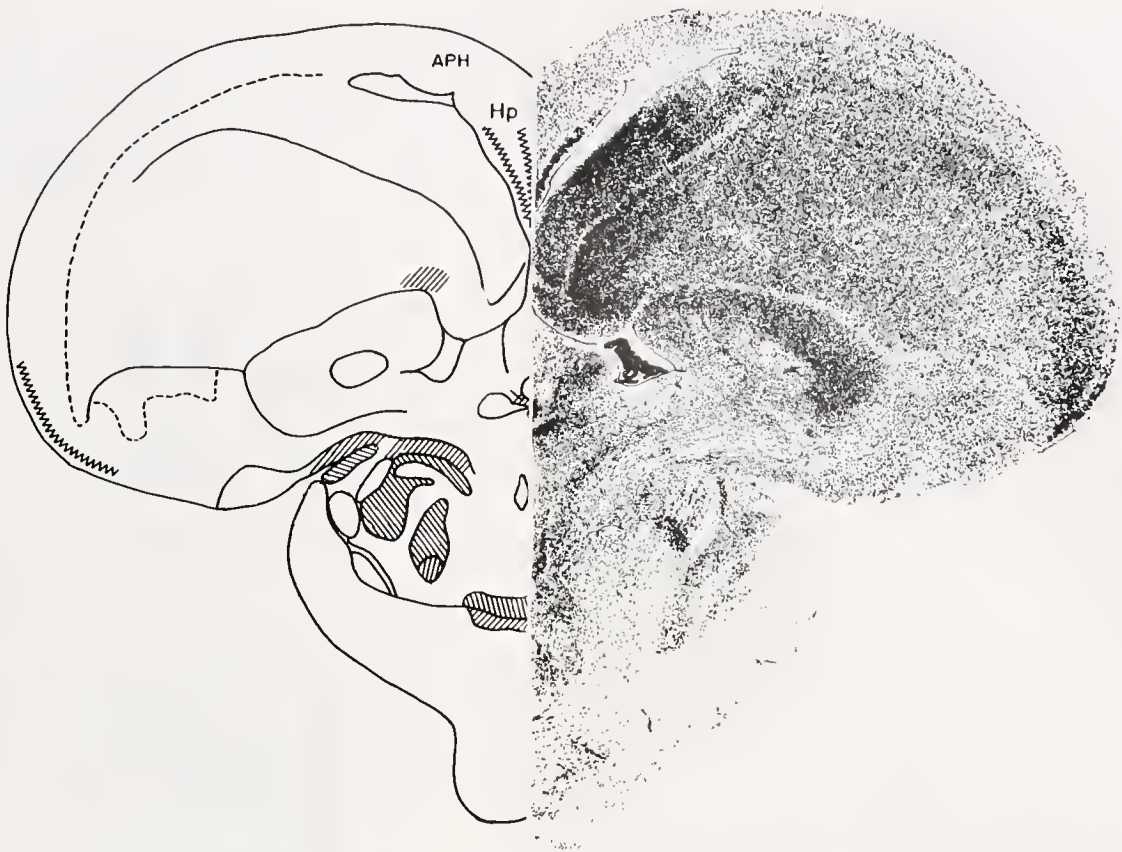


Figure 2. Diagram of a coronal cross-section of the hippocampal formation (HP, APH) in the laboratory pigeon. The right side of the figure shows the Nissl-stained brain section; the left side of the figure shows the boundaries of brain regions. The hippocampus (HP) and area parahippocampalis (APH) are labeled. Adapted from Karten and Hodos (1967).

tion bouts. Most remarkably, the changes in dendritic structure occur within 2 h of a squirrel's arousal from torpor, and it is suggested that such changes must therefore occur repeatedly throughout hibernation, with cyclic changes occurring with each bout of spontaneous activity (Popov and Bocharova, 1992; Popov *et al.*, 1992). Of course, it is possible that this remarkable process was occurring throughout the squirrel brain; other regions were not assessed in these studies. Future work should address this question, but nonetheless, such plasticity does exist in the hippocampus.

This plasticity may well be hormonally mediated because gonadal, thyroid, and adrenal hormones produce similar changes in the dendritic arbor of hippocampal pyramidal neurons in the laboratory rat (McEwen *et al.*, 1991). The hippocampus is, in fact, a remarkably plastic brain structure. As in the song nuclei of passerine birds, new neurons continue to be recruited in the granule layer of the dentate gyrus of the hippocampus in the adult laboratory rat (Altman and Bayer, 1990), and these new neurons make functional connections (Kaplan and Bell,

1983). But perhaps it is not a coincidence that such plasticity, rare in the adult brain, happens to be found in the hippocampus, a structure that plays such an important role in the neurobiology of learning.

Seasonal changes in spatial behavior and hippocampus in mammals. Evidence suggests that seasonal patterns of spatial behavior are linked to seasonal changes in the hippocampus. Voles, small rodents in the genus *Microtus* (subfamily Arvicolinae, family Muridae), are ideal subjects with which to test hypotheses on the evolution of cognitive adaptations, in particular the cognitive consequences of different mating systems (Gaulin and FitzGerald, 1986, 1989; Kavaliers *et al.*, 1993) and their underlying neural bases (Insel and Shapiro, 1992; Jacobs *et al.*, 1990; Shapiro *et al.*, 1991; Winslow *et al.*, 1993). Spatial learning, in particular, has proved an excellent model. Monogamous species, such as pine (*M. pinetorum*) or prairie (*M. ochrogaster*) voles, show little or no sexual dimorphism in space use in the field (FitzGerald and Madison, 1983; Getz and Hofmann, 1986). In contrast, among polygamous species, such as meadow

voles (*M. pennsylvanicus*) and montane voles (*M. montanus*), breeding males typically utilize a much larger home range than do breeding females (Gaulin and FitzGerald, 1988; Jannett, 1981; Madison, 1980). The presence or absence of sex differences in space use is correlated with similar patterns in spatial learning: sex differences with a male advantage (male performance superior to female performance) are present in polygamous meadow voles (Gaulin and FitzGerald, 1986, 1989; Kavaliers *et al.*, 1993), but sex differences are absent in monogamous voles (Gaulin and FitzGerald, 1986, 1989).

These patterns of space use are seasonal, however. Over the four seasons, polygamous meadow voles show marked changes in social system and space use (Gaulin and FitzGerald, 1988; Madison and McShea, 1987). In the winter, male home range decreases to a size similar to that of nonbreeding males or females; this is accompanied by an increase in social tolerance and the formation of mixed sex and lineage groups (Madison and McShea, 1987).

These behavioral changes are correlated with changes in brain structure. Under natural conditions, voles show large seasonal fluctuations in cranial volume and brain weight (Dehnel, 1949; Yaskin, 1984; Yaskin, 1989). These measures reach a maximum during the summer breeding season and a minimum in winter. The structural changes appear to be triggered by photoperiod, *i.e.*, the number of daylight hours. In the laboratory, meadow vole males reared under summer photoperiod (14 h daylight) had heavier brains than males reared under winter photoperiod (10 h daylight) (Dark *et al.*, 1987, 1990). Rearing photoperiod had no effect on normal females, although females masculinized with neonatal testosterone injections also showed this effect of photoperiod on adult brain size (Whaling *et al.*, 1990). Photoperiod thus appears to be the proximate cue triggering changes in brain mass, and the response appears to be sexually dimorphic.

Such drastic changes in spatial and social ecology, accompanied by gross changes in brain volume, might be expected to modulate spatial learning ability. This has now been shown in two related species, montane voles (*Microtus montanus*) and the deer mouse (*Peromyscus maniculatus*), both of which show sex differences in natural space use (Jannett, 1981; Galea *et al.*, 1994). Rodents reared under long (*i.e.*, summer) daylengths show sex differences in spatial learning, with a male advantage, on the Morris water maze (Morris, 1984), a task that yields consistent sex differences in laboratory meadow voles (Kavaliers *et al.*, 1993). However such differences were absent in rodents reared in short (*i.e.*, winter) daylengths (Galea *et al.*, 1994; Jacobs *et al.*, unpub.).

Data on spatial learning ability in wild-caught meadow voles (*M. pennsylvanicus*) suggest that such

differences develop in response to lengthening photoperiod. Wild meadow voles captured during natural short days (early December) were housed in the laboratory under long-day conditions (14 h daylight). After several weeks of habituation under the longer photoperiod, they were tested on a series of seven symmetrical mazes (Dav-

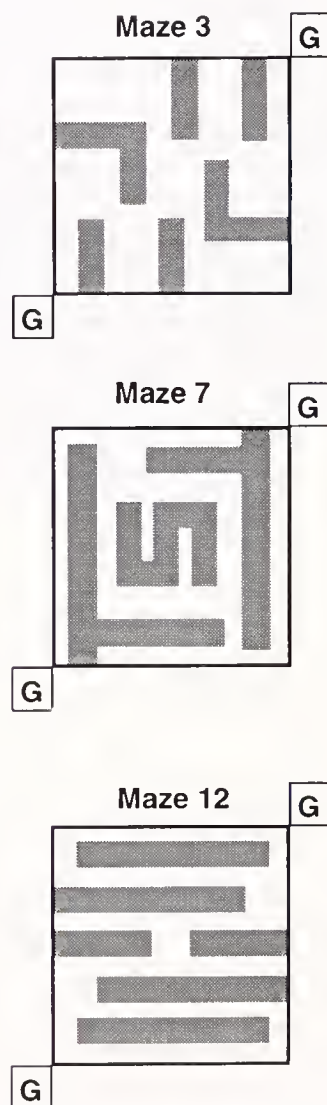


Figure 3. Schematic of symmetrical maze design, showing three of the seven maze configurations used to test meadow voles. Adult male and female voles were trapped in western Pennsylvania in late fall and brought to the laboratory. Here they were housed under long (14:10) photoperiods. Once habituated to the laboratory, food-deprived voles were required to shuttle between the two goal boxes (indicated by 'G') to obtain a food pellet. Each vole was allowed to shuttle back and forth in the maze until it had completed three error-free runs or until it had completed 12 runs. Each vole was tested only once per maze, and test periods were separated at intervals of several weeks. Performance was measured as the number of errors made; an error was defined as an entry into a blind alley. Further methodological details can be found in Gaulin and FitzGerald (1989).

enport *et al.*, 1970; Fig. 3), at intervals of approximately 1 month (details in Gaulin, 1995). As testing proceeded on the series of mazes, the voles had thus been housed for progressively longer periods under summer photoperiods. The voles initially showed no sex differences in maze performance; however, after several months under long-day conditions, a sex difference in performance indeed developed (Gaulin, 1995; Jacobs *et al.*, unpub.; Fig. 4).

Exposure to an increased photoperiod produces significant increases in brain and body weight in meadow voles, a response that is more pronounced in males (Dark *et al.*, 1990). Therefore, one might expect that not only brain weight but also hippocampal weight would increase or decrease predictably with season, and that this response would be more pronounced in males. Vladimir Yaskin has demonstrated such a specific response in small mammals, including four species of voles and two species of shrews, in various sites in the former Soviet Union. In wild-trapped voles and shrews, many parts of the brain are larger in the summer than during the winter. The greatest relative change between seasons in the size of a major brain structure, as measured by either dry or wet weight, is seen in the hippocampus. Other structures that were measured included neocortex, striatum, cerebellum, and olfactory bulbs. In shrews, the

average adult hippocampal weight is 19.7% lighter in animals trapped in the fall than in those trapped the preceding summer, and 25.6% heavier in the spring-trapped adults than in adults trapped in the winter. Moreover, this change is sexually dimorphic both in shrews and voles, showing a greater increase in males in the spring, as would be predicted from concomitant increase in space use by males during the breeding season (Yaskin, 1984; Yaskin, 1994).

An important point to address, however, is that we do not know what savings in metabolic cost are created with decreases in specific brain structures, such as the hippocampus. In addition, the metabolic cost of the tissue itself (which may weigh only a few milligrams) is the tip of the iceberg. It is the cost of the behavior that is expensive. Thus the cost of increasing the volume of song nuclei is small; the cost of using these nuclei to attract mates is high. Consequently we must view the system as a whole and ask: what is the combined cost of this behavioral plasticity? This is the cost that influences the course of natural and sexual selection; what is surprising is that certain brain structures, such as the song nuclei and the hippocampus, are unusually plastic, as are the behaviors they mediate.

Reducing foraging costs

There are at least two good ways to reduce the cost of foraging in the winter. The first method is to escape a cold climate by migrating to a warmer one. Not surprisingly, migration is a strategy frequently adopted by flying animals such as birds and insects (Alerstam, 1990). Migration allows an animal to forage all year under summer conditions of temperature and food abundance, and thus one might expect no change in learning ability. However, the actual migration may require special spatial learning abilities. Homing pigeons, for example, have larger hippocampi than other pigeon strains, and the hippocampus is used for sun-compass orientation and for recognition of the home loft (Bingman, 1990). There is also recent evidence that passerine bird species that migrate have relatively larger hippocampi than species that do not migrate, when other ecological and phylogenetic factors have been accounted for (Healey, pers. comm.). Thus migration may require seasonal changes in cognitive abilities, such as spatial orientation, and may be accompanied by seasonal changes in the hippocampus.

The strategy of food-storing. Changes in hippocampal structure and spatial behavior are indeed seen in bird species that employ a different strategy to reduce foraging costs: storing food. Food-storing is a common strategy both in temperate-zone and tropical-zone faunas, to redistribute food surplus more evenly in time. Often the food is redistributed more evenly in space as well. For

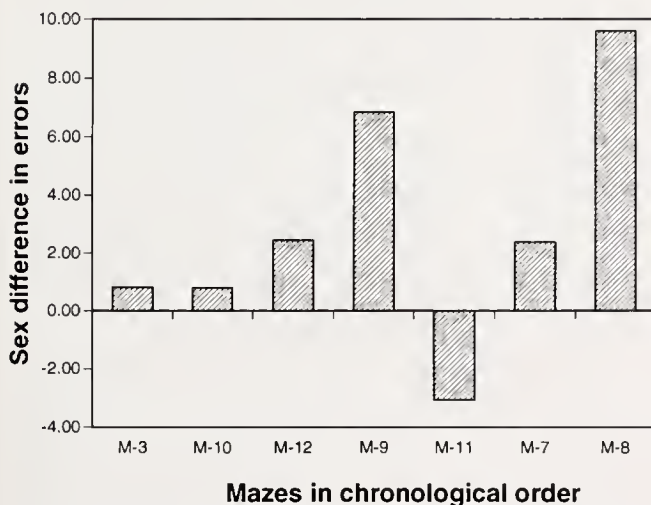


Figure 4. Changes in the sex difference in symmetrical maze performance in wild-caught meadow voles. Sex difference in maze performance is shown for a series of seven mazes. Maze performance is ordered chronologically; *i.e.* shown in the order in which voles were tested. Thus performance on the first and last maze was separated by about 7 months. Sex difference in performance was estimated as the difference (female minus male) in errors between males and females ($n = 5$ females, 4 males). There was a significant increase in the size of the male advantage in performance over the course of testing, probably related to the increased time spent housed under long photoperiods (Gaulin, 1995; Jacobs *et al.*, unpub.).

example, a gray squirrel (*Sciurus carolinensis*) may harvest 10,000 hickory nuts and cache these nuts one by one over an area of a few hectares. The squirrel then may use spatial memory to retrieve these caches (Jacobs and Liman, 1991). The use of spatial memory to retrieve caches is correlated with increased hippocampal volume in both birds and mammals (Jacobs and Spencer, 1994; Sherry *et al.*, 1992). Hence one might predict that there would be seasonal changes both in the behavior of food-storing in this manner and in the hippocampus, a structure necessary for the retrieval of food caches (Sherry and Vaccarino, 1989).

Seasonal changes in the hippocampus in food-storing birds. Recent evidence suggests that seasonal changes in hippocampal structure and function do occur in food-storing birds. In the fall, black-capped chickadees (*Parus atricapillus*) begin storing food in scattered locations throughout their group territory. In the laboratory, such caches are retrieved using spatial memory of locations, an ability requiring the hippocampus; chickadees with hippocampal lesions can no longer locate their food caches, although they retain other types of spatial information (Sherry and Vaccarino, 1989). The behavior of scatter hoarding itself appears to be triggered by changes in photoperiod. Wild-caught chickadees store more intensely under autumn photoperiod conditions than under spring conditions (Shettleworth *et al.*, 1995).

The seasonal modulation of food-storing behavior is paralleled by seasonal changes in hippocampal structure. Wild black-capped chickadees trapped throughout the year show a seasonal pattern in hippocampal volume, relative to the volume of the telencephalon. The greatest volume is seen in October (Smulders *et al.*, 1995). During this period, chickadees start storing seeds intensely throughout their newly expanded home range. Since increased food-storing behavior is correlated with increases in hippocampal size in young parids (Clayton and Krebs, 1995), this suggests that a similar plasticity may be seen in adults. The change in volume is also accompanied by an increase in neurogenesis in the chickadee hippocampus (Barnea and Nottebohn, 1994). The increase in neurogenesis also peaks in October, suggesting that the behavioral changes during this period are key to understanding plasticity of the adult hippocampus.

Mammalian food-storers, such as gray squirrels, also show seasonal cycles in storing behavior in the field, although not in captivity (pers. obs.). In contrast, chickadees are seasonal both in the field and in the laboratory, even under constant photoperiod (Sherry, pers. comm.). Hence it is unclear whether scatter-hoarding mammals, such as gray squirrels, would show the same changes in hippocampal size as do black-capped chickadees. In addition, male squirrels greatly increase their home range in the winter during the breeding season, which should

also be correlated with increases in hippocampal size. Seasonal changes in hippocampal size might be explained by two selective pressures: to increase the efficiency of food-storing and to increase the efficiency of breeding behavior (Jacobs, 1996). Such a pattern of combined sex and species differences in the hippocampus has already been found in kangaroo rats (Jacobs and Spencer, 1994). I am currently testing this idea by measuring hippocampal size in scatter-hoarding rodents collected at different seasons.

Conclusion

One might say that there are only two ways to face the winter: reduce costs by reducing the need to forage by opting for a more sedentary lifestyle with less cognitive capacity, or reduce costs by redefining the game, by migration or food-storing. These latter tactics appear to require more movement, more cognitive processing, and hence a larger investment in brain structure, which, it appears, can be 'bought' with the new savings in foraging costs.

Although these patterns of seasonal changes in brain structure challenge our notion of how a brain is supposed to behave, perhaps this notion has a historical explanation: most neuroscience research is conducted on animals housed under constant photoperiod. Thus the remarkable plasticity seen first in canaries and now in small rodents and food-storing birds may not be remarkable at all, but quite commonplace among species that live in seasonal environments. Seasonal changes in spatial learning have even been documented in humans, correlating with annual cycles of testosterone in normal men (Kimura and Hampson, 1994) and in patients suffering from seasonal affective disorder; these patients show deficits in spatial cognition tasks (O'Brien *et al.*, 1993). Phenotypic plasticity in learning ability and brain structure may well be a general phenomenon; it already appears to have clinical correlates in humans.

For many reasons, then, the phenotypic plasticity of learning is a problem worth further study. The implications for behavioral ecology are functional: the behavior of a winter and a summer individual may be adapted to different ecological conditions; foraging decisions in one season may be optimized relative to different cognitive capacities. Or, males or females within a species could show enhanced abilities to track resources depending on season, which would allow them to forage more efficiently than other age-sex-season classes. Future generations of foraging models might need to incorporate seasonal changes in neural and cognitive capacity to accurately describe the behavior of a species. In any case, there is no doubt that an interface is growing between cognitive neuroscience and behavioral ecology—one

that may soon change neuroscientists' ideas about the brain and its limits and capacities, as well ecologists' notions about the plasticity and evolution of complex behaviors.

Acknowledgments

I would like to acknowledge Steve Gaulin's invaluable advice, help and support in our studies of seasonal changes in behavior; Candace Hisatake for technical assistance; Eric Bittman, John Dark and Irving Zucker for their critical discussion of these ideas; and two anonymous reviewers for their valuable comments on the manuscript. Supported by NSF IBN-9307317.

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Neuroethology of Orientation and Navigation: Introduction

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Vince Dethier would have appreciated the vertebrate studies that were presented in this symposium. Vince was a keen observer of animals, the key to success in neuroethology.

The first of the papers that follow (Kawasaki, 1996) describes work with an animal model introduced by the late Walter Heiligenberg, another animal observer *par excellence*. Two phylogenetically unrelated groups of fish use weak electrical signals to communicate, forage, navigate, and orient at night. One group occurs in South America and the other group in Africa. The signals of many of the South American electric fish are cyclical waves. The frequency of these waves varies from species to species. *Eigenmannia* is the best studied “wave” fish. When two *Eigenmannia* fish having slightly different wave frequencies encounter one another, the fish with the higher frequency raises its frequency further, and the fish with the lower frequency lowers its further. As a result, the difference in signal frequency between the two becomes greater than before. This is the jamming avoidance response. As the name implies, this maneuver prevents fish from disturbing each other in their exploration of the environment. Walter and his colleagues, including Masashi Kawasaki, figured out how *Eigenmannia* determines the sign of frequency differences. *Eigenmannia* does not have any internal reference by which it can compare its own frequency with the frequency of another fish. When two sinusoidal waves of different frequencies are mixed, a beat waveform results. *Eigenmannia* uses differential changes in the phase and amplitude of the beat waveform between different body surface areas to determine the sign of frequency differences.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

Kawasaki made the remarkable discovery that *Gymnarchus niloticus*, one of the African wave species, uses the same computational algorithm as *Eigenmannia* to determine the sign of frequency differences. This finding is surprising for two reasons. (1) These two groups are phylogenetically unrelated. (2) Other African species belonging to the same group use an “efference copy” to compare the fish’s own frequency and the frequency of another fish. An efference copy is a copy of neural signals from the medullary pacemaker nucleus that drives the electric organ. The efference copy is sent to neurons that compare it with external sensory signals. Kawasaki found that the neural circuits for the transmission of efference copies from the pacemaker nucleus are missing in *Gymnarchus*. Even more fascinating is his finding that *Eigenmannia* and *Gymnarchus* use different brain areas to do similar neural computations. The sign of phase difference between two body areas is detected by neurons in the torus semicircularis in the midbrain in *Eigenmannia* and in the medullary electrosensory lateral line lobe in *Gymnarchus*. Kawasaki’s work beautifully illustrates the power of the comparative approach in neuroscience.

The ability to entice animals to perform natural behavior in controlled conditions of the laboratory is another key to success in neuroethology. For many years Vince Dethier used the famous proboscis extension response to investigate the taste responses of flies. Jim Simmons was the first to succeed in training bats to perform auditory perceptual tasks such as discriminating between two objects located at different ranges. The bats emitted echolocating pulses toward two objects located at different angles and distances, and when they pointed their heads in the direction of the nearer object they were rewarded with a mealworm. Bats quickly learned to point to the nearer of the two objects at whichever angle it occurred. This technical feat was possible because Simmons knew his animals. He introduced another revolu-

tionary tool to use with the training method. He registered outgoing sonar pulses near the bat's nose, manipulated them on a computer, and delivered the modified sound back to the bat. The manipulations included changing the spectral properties and echo delays.

These two technical advances made it possible to investigate bat echolocation in the laboratory. Using these methods, Simmons established that big brown bats (*Eptesicus*) can detect small shifts in both the frequency of spectral peaks and the timing of echoes. As Simmons points out in his paper (1996), these bats can detect time shifts as small as 10–15 ns. Hyperacuity is well known in the visual and auditory systems. For example, man can perceive a time difference of 10 μ s between the ears. But how can we explain this phenomenon in terms of the activities of individual neurons when a nerve impulse is 100-fold longer? Simmons and his associates noted that

the electrical potentials recorded from a cluster of neurons resembled the train of simulated FM pulses and their echoes delivered to the bat. These neural responses were a time-expanded version of the stimulus. Thus Simmons thinks that the bat solves the problems of hyperacuity by time-expansion. He will have to show that the bat uses these neural response patterns for encoding temporal information carried by echoes.

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Comparative Analysis of the Jamming Avoidance Response in African and South American Wave-Type Electric Fishes

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Abstract. African wave-type electric fish, *Gymnarchus*, and South American wave-type electric fish, *Eigenmannia*, have evolved electrosensory and electromotor systems independently. Nevertheless, they exhibit a similar electrical behavior, the jamming avoidance response (JAR). When two individuals with slightly different frequencies of electric organ discharge (EOD) meet, they shift discharge frequencies away from each other to avoid mutual jamming of their electrolocation systems. These two genera of electric fishes perform this behavior using an identical set of complex computational rules. Reflecting their independent evolution, however, neuronal implementation of the computational steps appears to take different forms. One of the essential computational steps, phase comparison, is performed in the hindbrain in *Gymnarchus* and in the midbrain in *Eigenmannia*. The comparison of these two species in this paper revealed an example of how different brain structures perform functionally similar tasks in independently evolved systems that have a similar overall behavioral function.

Introduction

Weakly electric fishes generate a weak electric field on the order of millivolts per centimeter from their electric organ. The electric field envelops the fish, and the distortion of the electric field caused by environmental objects

is sensed by electroreceptors located all over its skin surface. This active electrolocation process helps the fish to locate and identify surrounding objects and prey (Bastian, 1986). As with detection mechanisms in other sensory modalities, the electrosensory system seems to employ temporal and spatial information for localizing and identifying objects. The complexity of the temporal and spatial analysis by central electrosensory mechanisms has been revealed in a particular electromotor behavior, the jamming avoidance response (JAR) (Heiligenberg, 1991). This behavior is not an electrolocation behavior *per se* but preserves electrolocation ability when the fish's own electric organ discharges (EODs) are jammed by those of a neighboring fish.

Similar JARs are known in remotely related electric fishes, *Gymnarchus* and *Eigenmannia* (Bullock *et al.*, 1975). *Gymnarchus* and *Eigenmannia* are wave-type electric fishes that emit EODs at constant, individually fixed frequencies (250–600 Hz). When two fish with similar discharge frequencies meet, however, their electrolocation systems jam each other, resulting in a partial loss of electrolocation ability. To avoid this jamming, the two fish shift their discharge frequencies in the direction that will increase the frequency difference, restoring their electrolocation ability. The fish decide whether they should increase or decrease their EOD frequency according to the *sign* of frequency difference between their own and the neighbor's EOD (Watanabe and Takeda, 1963; Bullock *et al.*, 1972, 1975).

Gymnarchus, an African mormyrid fish, and *Eigenmannia*, a South American gymnotiform fish, are distantly related electric fishes that lack common electroreceptive ancestors and appear to have evolved both electroreception and electrogenesis independently (Lauder and Liem, 1983). Therefore, these two genera provide a

Received 30 November 1995; accepted 28 February 1996.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

Abbreviations: EOD: electric organ discharge; ELL: electrosensory lateral line lobe; JAR: jamming avoidance response.

rare opportunity to study complex neuronal mechanisms in vertebrates that have independently evolved to perform a similar behavior. In this article I compare the JARs of *Gymnarchus* and *Eigenmannia* in terms of computational rules, physiology, and anatomy.

Computational Rules

Despite their independent evolution, *Gymnarchus* (Kawasaki, 1993) and *Eigenmannia* (Heiligenberg *et al.*, 1978; Heiligenberg and Bastian, 1980) share a remarkably similar but complex set of computational rules for their JAR that has the following four major computational features.

The first shared feature is that the sign of the frequency differences is extracted from complex afferent information without referring to pacemaker signals internally. The pacemaker nucleus is the intrinsic neuronal oscillator in the medulla in which neurons fire in synchrony, producing command pulses that are sent to the electric organ and trigger individual EOD waves. Thus, the fish could compare the frequency of the pacemaker signal with that of a neighbor's discharge to obtain information about the sign of frequency difference between its own and the neighbor's discharge. The following experiment, however, eliminates this possibility. The fish's EOD was silenced by curare, and an external electrical signal that mimics the fish's natural EOD was applied to the fish. The frequency of the replacement signal could be arbitrarily set independent of the frequency of the pacemaker signal. EODs of a neighbor were mimicked by the second external signal. JAR was driven by the frequency difference between these two external signals regardless of the frequency relation between the pacemaker and the neighbor's signal, demonstrating that fish do not make internal reference to the pacemaker for the JAR (Heiligenberg *et al.*, 1978; Kawasaki, 1993).

The lack of internal reference to the pacemaker in *Gymnarchus* is surprising because it belongs to the same family as *Gnathonemus*, a pulse-type African electric fish (Hopkins, 1986) that possesses the internal reference mechanisms. In *Gnathonemus*, the command nucleus in the medulla, which generates the EOD rhythm and drives the electric organ, sends a copy of the electromotor command, a corollary discharge, within the brain to the sensory systems in order to gate all afferent signals. Thus, exafference, the sensory input due to a stimulus caused by an external source, and reafference, a sensory input caused by the animal's own acts, are clearly distinguished by this neuronal hardware (Meyer and Bell, 1983; Bell, 1986a, b; Bell and Grant, 1989). The anatomical organization of the pacemaker nucleus of *Gymnarchus* is similar to that of *Gnathonemus* but lacks the cor-

ollary discharge projection. A projection from the pacemaker nucleus in *Gymnarchus*, which resembles the corollary discharge pathway in *Gnathonemus*, does not reach any sensory areas of the brain and solely projects back to the relay nucleus that drives the electric organ (Kawasaki, 1994).

The second computational feature found in both *Gymnarchus* and *Eigenmannia* is that the sign of the frequency difference between the fish's own EOD and the neighbor's EODs is computed from the temporal pattern of amplitude and phase modulation that is created in the summed signal of the two EODs. Each of the amplitude and phase modulations occurs at the *absolute* frequency difference between the fish's own and a neighbor's EOD and thus cannot encode the sign of the frequency difference. The temporal combination of them, however, uniquely encodes the sign of the frequency difference, and behavioral experiments demonstrate that fishes use this temporal pattern of amplitude and phase modulation for the JAR (Fig. 1) (Heiligenberg *et al.*, 1978; Kawasaki, 1993).

The third shared feature is the use of phase *difference* for computing the time course of phase modulation. Analysis of phase modulation requires a timing reference signal. The pacemaker signal, which is constant in phase, would be an ideal timing reference signal and could be compared for detecting phase modulation of the sensory signal. The first computational rule, however, eliminates this possibility. Instead, fish compute phase *difference* between different body areas to detect the time course of phase modulation (Fig. 2, left). Experimental elimination of the phase difference between body areas eliminates the JAR (Heiligenberg and Bastian, 1980; Kawasaki, 1993).

The last shared feature is the distributed analysis of sensory information. These fish always perform the JAR correctly—that is, they shift the EOD frequency in the direction that increases the frequency difference between two fish, regardless of the spatial orientation of the electric field by a neighbor. Any single computational component associated with a particular area of the body surface can encode the sign of the frequency difference only ambiguously, however, because the computation of phase difference inherently makes errors when the spatial orientation of a neighbor's electric field changes. The right panel of Figure 2 explains how differential phase computation errs in detecting the correct phase modulation. Although phase computation in each area may or may not provide correct information, it is differentially weighted by the size of the local amplitude modulation. Totalling such computational results from all body areas, the fish is always able to perform the JAR correctly (Heiligenberg and Bastian, 1980; Kawasaki, 1993). Neuronal mechanisms for this spatial integration of information remain to be explored.

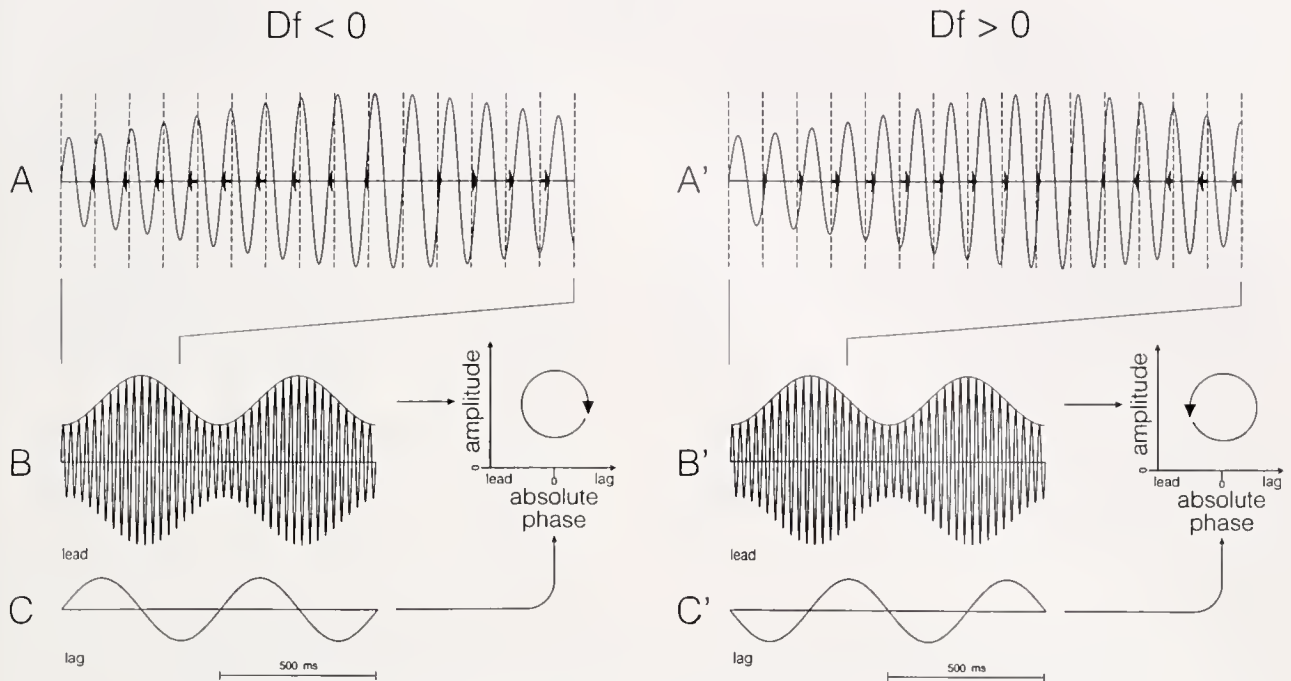


Figure 1. Amplitude and phase modulation in the mixture of two sinusoidal signals mimicking the fish's own and a neighbor's EODs. The frequency difference is $Df = -2$ Hz on the left panel and $Df = +2$ Hz on the right panel. Amplitude, or envelope of the signal mixture (B and B'), shows sinusoidal modulation at 2 Hz. The first part of B and B' is expanded in time in A and A' to reveal the phase modulation. The phase leads that of the uncontaminated signal (vertical broken lines) during amplitude rises and lags during amplitude falls in A. In contrast, timing of zero-crossings lags during amplitude rises and leads during amplitude falls in A'. In C and C', zero-crossing timing of B in reference to that of the uncontaminated signal (absolute phase) is plotted. In the amplitude-phase plane (inserts), the amplitude (*i.e.*, the envelope of B and B') is plotted on the ordinate, and the absolute phase (C and C') is plotted on the abscissa. Due to unique temporal relations between amplitude and phase modulation for $Df < 0$ and $Df > 0$, a circular graph rotates clockwise and counterclockwise for $Df < 0$ and $Df > 0$, respectively. For graphical purposes, signal frequency is set at 40 Hz instead of the natural range of 250 to 600 Hz, and the amount of phase modulation, which is too small to be recognized in B, is exaggerated in A. (After Kawasaki, 1993.)

Phase Comparison Mechanisms

It is remarkable that *Gymnarchus* and *Eigenmannia* have independently evolved the JAR with identical computational rules of this complexity. Because of their independent evolution, however, the physiological implementation of these computational steps is not necessarily expected to be identical. Although physiological mechanisms for the JAR in *Eigenmannia* are well analyzed (Bastian and Heiligenberg, 1980; Heiligenberg and Rose, 1985, 1986; Rose and Heiligenberg, 1986; Rose *et al.*, 1988; Kawasaki and Heiligenberg, 1990), analysis of those of *Gymnarchus* has only recently begun (Kawasaki and Guo, 1996). So far, comparison of the physiological mechanisms for the detection of phase difference is possible.

Differential phase comparison in *Gymnarchus*

Because *Gymnarchus* emits EOD at a constant frequency, the timing of zero-crossings, or phase, of electro-

sensory feedback of EODs at electroreceptors is constant when there is no neighboring fish. When a neighbor appears, however, electroreceptors are stimulated by the sum of the EODs of the fish and its neighbor; these modulate in phase as well as in amplitude at a frequency equal to the absolute frequency difference between the two fish (Fig. 1). Because of the geometrical difference between the fish's own electric field and that of the neighbor, the amplitude ratios between them are different at different locations on the body surface. This differential contamination results in a difference in the depth of phase modulation, which is a function of the ratio between the two signals (Fig. 2). This phase difference, one of the essential cues for the JAR, is detected by the following physiological mechanism.

The S-type tuberous receptor afferents fire one action potential at the zero-crossing of each stimulus cycle and thus encode phase by the timing of the action potentials. They project directly to the inner cell layer of the medial zone in the electrosensory lateral line lobe (ELL).

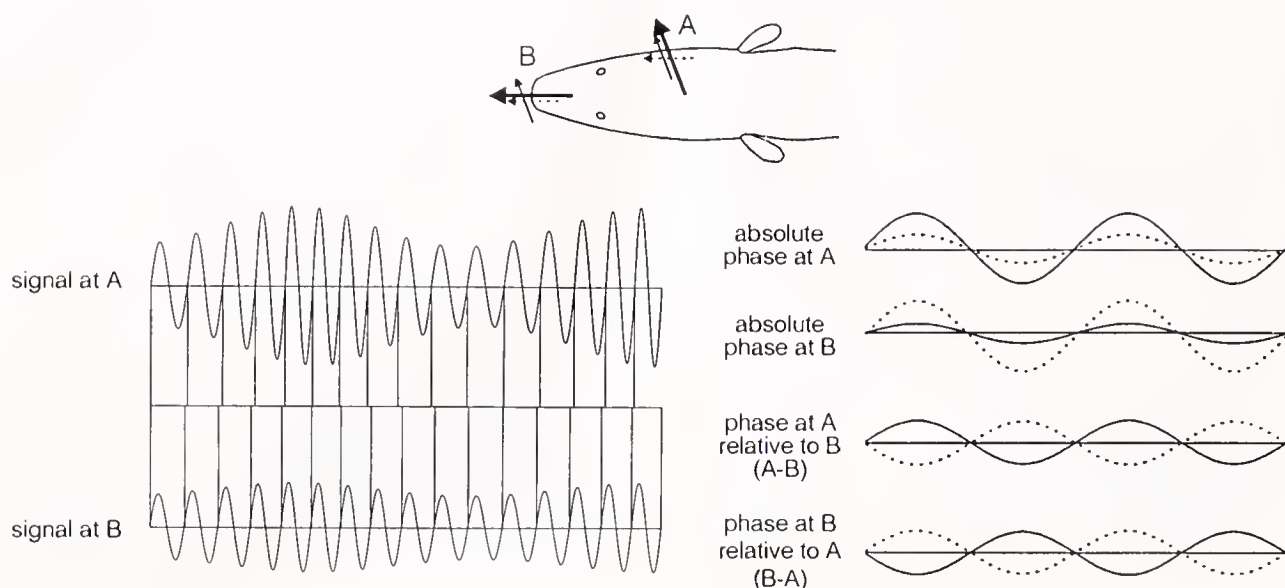


Figure 2. (Left) Phase differences between two body areas A and B. The fish's own electric organ discharge establishes a radial electric field (long arrows) because of the internal location of the electric organ, while the neighbor's EOD creates a more-or-less parallel field (short solid arrows) due to the external location of the neighbor's electric organ. Electroreceptors, which are sensitive to the local electric field oriented perpendicularly to the skin surface, are more-or-less evenly stimulated by the fish's own EOD across all body areas. The degree of stimulation by the neighbor's EOD, however, differs between areas A and B. The depth of phase modulation is a function of the ratio between the two signals; thus differential phase modulations arise between areas A and B. (After Kawasaki, 1993.) (Right) Differential phase computation may yield a wrong result depending on the spatial orientation of the neighbor's electric field. Two spatial orientations of the neighbor's electric field are assumed (short solid arrows and broken short arrows in left). The vertical axis represents absolute and relative phase modulations at areas A and B. The solid orientation yields a deeper phase modulation in A, and the dotted orientation yields a stronger modulation in B because of the different mixing ratios. Subtraction of the absolute phase at A from that of B, or *vice versa*, is shown in the bottom two traces. Note that the change of orientation results in the inversion of the sign of the phase difference. Note also that such a sign inversion of phase information implies the opposite sign of frequency difference between the fish's own and the neighbor's EOD as evident from Fig. 1.

Branches of these afferent fibers project also to the somata of giant cells in the medulla (Fig. 3). The giant neurons in turn project bilaterally to the inner cell layer of the ELL. Giant cells also fire one action potential for each stimulus cycle in response to the input afferent fibers. Thus, at the inner cell layer of the ELL, phases at different locations on the body surface are jointly represented by the timing of action potentials by S-type afferent fibers and giant cells. Neurons in the inner cell layer respond to phase *difference*.

Figure 4 shows responses of differential-phase-sensitive neurons in the ELL recorded as extracellular potential. The fish was placed in a chamber in which the head and trunk portions of the body surface were electrically isolated for accurate control of phase difference between the two areas. When the phase of the head portion was modulated by $\pm 70 \mu\text{s}$ at 1 Hz and the trunk portion was stimulated with an unmodulated signal with the same carrier frequency, the neurons responded to phase *advance* of the

head signal. When the stimuli in the head and the trunk were interchanged, the neuron responded to the phase delay of the trunk stimulus, demonstrating that the neuron responds to the phase difference between the head and trunk areas. Intracellular recordings and labeling revealed that these neurons project to the torus semicircularis in the midbrain (Kawasaki and Guo, 1996).

These differential-phase-sensitive neurons are sensitive to a phase difference in the microsecond range. Behavioral and physiological experiments demonstrate that *Eigenmannia* can resolve sub-microsecond phase differences, and corresponding neuronal sensitivities are found in central neurons (Rose and Heiligenberg, 1985; Kawasaki *et al.*, 1988). Behavioral thresholds for phase comparison have not been measured in *Gymnarchus*.

Differential phase comparison in Eigenmannia

Similarly to the S-type tuberous electroreceptors in *Gymnarchus*, T-type tuberous electroreceptors in *Eigen-*

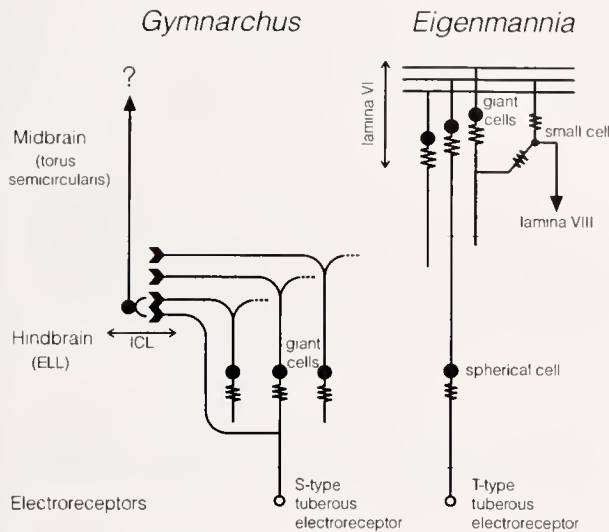


Figure 3. Comparison of differential phase coding systems in *Gymnarchus* and *Eigenmannia*. S-type and T-type electroreceptor afferents, giant cells, and spherical cells fire an action potential for one stimulus cycle, thus encoding absolute phase at each electroreceptor organ. Neurons in the inner cell layer (ICL) in the ELL in *Gymnarchus* and small cells in lamina VI of the torus semicircularis in the midbrain in *Eigenmannia* receive such phase-locked inputs from different body areas and respond to the phase difference between them. The nature of the synapses in *Gymnarchus* is not known. Synapses represented by the resistor notation in *Eigenmannia* are of mixed type.

mannia fire one action potential for each stimulus cycle encoding phase information (Scheich *et al.*, 1973). Their sole projection is made to the somata of spherical cells in the ELL. The response of the spherical cells is also phase preserving, responding to each cycle of electrosensory stimulus by an action potential. Unlike in *Gymnarchus* in which the first central neurons, the giant cells in the ELL, bilaterally spread their axons for differential phase computation within the ELL, the spherical cells in *Eigenmannia* do not spread any processes within the ELL—they project single axons to lamina VI of the torus semicircularis in the midbrain. There they synapse onto the somata of the giant cells that then spread large axonal arbors for differential phase comparison. The small cells in the lamina VI receive inputs from the giant cells and the spherical cells and respond to the phase difference between these inputs (Fig. 3) (Carr *et al.*, 1982, 1986a, b; Heiligenberg and Rose, 1985).

Comparative Implication

Thus, the function of differential phase comparison, one of the essential computational elements for the JAR, is assigned to different brain structures in *Gymnarchus* and *Eigenmannia*. Despite this difference, the internal organization of the phase comparison circuitry within

the structures is strikingly similar. In both systems, absolute phase information is supplied to phase-comparing neurons (neurons in the inner cell layer of the ELL in *Gymnarchus* and small cells in the lamina VI of the torus semicircularis in *Eigenmannia*) via two pathways—directly by phase coding afferent to the structure (S-type afferents in *Gymnarchus* and spherical cell afferents in *Eigenmannia*) and indirectly by adendritic giant cells with large axonal arbors. Also the differential-phase-sensitive neurons occur in a layered structure. The existence of similar phase-comparison circuits in different brain structures demonstrates that these fish indeed have developed this function by convergent evolution.

In *Gnathonemus*, which belongs to the same family (Mormyridae) as *Gymnarchus*, timing information of EOD pulses from neighboring fish appears to carry a species-specific or gender cue. The neighbor's EOD pulses are sampled by knollenorgan electroreceptors, and their afferent fibers project onto an adendritic soma of neurons in the nucleus of the ELL; this soma is reminiscent of the giant cells of *Gymnarchus*. Unlike the neurons in *Gymnarchus*, however, these neurons do not project within the ELL, they project to the midbrain (Szabo *et*

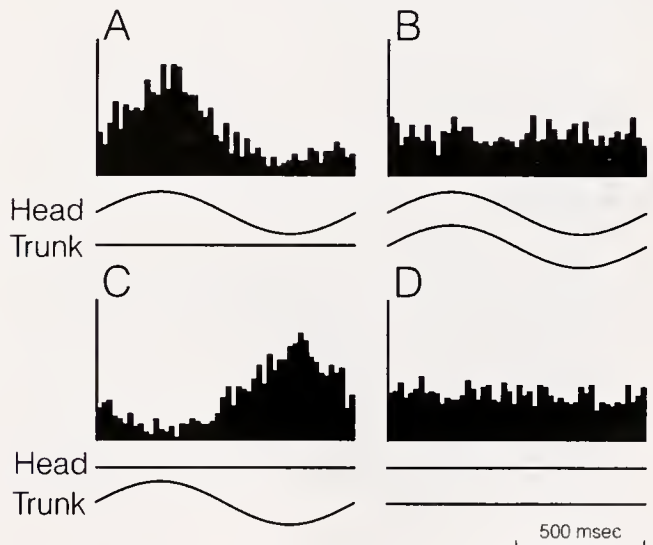


Figure 4. Extracellularly recorded single unit responses to phase difference in the ELL of *Gymnarchus*. Two traces below each histogram show the absolute phase of signals applied at the head and at the trunk part of an experimental chamber in which the curarized fish was placed for independent stimulation of these body areas (Kawasaki, 1993). (A) The trunk was stimulated with an unmodulated sinusoidal signal; the head was stimulated with a sinusoidal signal whose carrier frequency was identical but modulated in phase ($\pm 70 \mu\text{s}$). The neuron showed a strong response to phase advance in the head. (B) The head and the trunk received identical stimuli which modulated in phase as in the head in A. (C) Stimuli in A were swapped between head and trunk. The neuron responds when phase at the trunk is delayed. (D) Both head and trunk received unmodulated sinusoidal signals. Note that B and D are similarly indifferent. (After Kawasaki and Guo, 1996.)

al., 1975; Enger *et al.*, 1976; Mugnaini and Maler, 1987). Phase comparison appears to occur in the midbrain in this fish (Friedman and Hopkins, 1995).

The comparison of these differential phase circuits indicates that different brain structures may perform a similar function in independently evolved systems with similar overall function; conversely, homologous structures may not be assigned a similar function even in closely related species.

Acknowledgments

This study was supported by NIMH grant R29 MH48115-01A1 to M. K. I thank Yasuko Kawasaki for preparing the figures and Cameron McLaughlin for editing my English.

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Representation of Perceptual Dimensions of Insect Prey During Terminal Pursuit by Echolocating Bats

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Abstract. The echolocating big brown bat, *Eptesicus fuscus*, broadcasts brief frequency-modulated (FM) ultrasonic sounds and perceives objects from echoes of these sounds returning to its ears. *Eptesicus* is an insectivorous species that uses sonar to locate and track flying prey. Although the bat normally hunts in open areas, it nevertheless is capable of chasing insects into cluttered environments such as vegetation, where it completes interceptions in much the same manner as in the open except that it has to avoid the obstacles as well as catch the insect. During pursuit, the bat shortens its sonar signals and increases their rate of emission as it closes in to seize the target, and it keeps its head pointed at the insect throughout the maneuver. In the terminal stage of interception, the bat makes rapid adjustments in its flight-path and body posture to capture the insect, and these reactions occur whether the bat is pursuing its prey in the open or close to obstacles such as vegetation. Insects can be distinguished from other objects by the spectrum and phase of their echoes, and *Eptesicus* is very good at discriminating these acoustic features. To identify the insect in the open, but especially to distinguish which object is the insect in clutter, the bat must have some means for representing these features throughout the interception maneuver. Moreover, continuity for perception of these features is necessary to keep track of the prey in complex surroundings, so the nature of the auditory representations for the spectrum and phase of echoes has to be conserved across the approach, tracking, and terminal stages. The first problem is that representation of changes

in the phase of echoes requires neural responses in the bat's auditory system to have temporal precision in the microsecond range, which seems implausible from conventional single-unit studies in the bat's inferior colliculus, where the temporal jitter of responses typically is hundreds of microseconds. Another problem is that echoes do not explicitly evoke neural responses in the inferior colliculus distinct from responses evoked by the broadcast during the terminal stage because the delay of echoes is too short for responsiveness to recover from the emissions. In contrast, each emission and each echo evokes its own responses during the approach and tracking stages of pursuit. How does the bat consistently represent the phase of echoes in spite of these evident limitations in neural responses? Local multiunit responses recorded from the inferior colliculus of *Eptesicus* reveal a novel format for encoding the phase of echoes at all stages of interception. Changes in echo phase (0° or 180°) produce shifts in the latency of responses to the emission by hundreds of microseconds, an unexpected finding that demonstrates the existence of expanded time scales in neural responses representing the target at all stages of pursuit.

Introduction

Echolocating bats broadcast ultrasonic sounds and perceive objects from echoes that return to their ears (Griffin, 1958; Novick, 1977; Popper and Fay, 1995). Most species of echolocating bats are insectivorous and use their sonar to detect and locate flying prey as well as to locate and avoid obstacles (Fenton, 1995; Griffin, 1958; Neuweiler, 1990; Schnitzler and Henson, 1980; Simmons *et al.*, 1995a). During pursuit of prey, the bat transmits a series of sounds and perceives the insect and

Received 29 February 1996; accepted 9 April 1996.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

other objects as a series of representations or images that guide the interception maneuver to a successful capture. As the bat approaches the target, the delay of echoes shortens from one broadcast to the next. During the terminal stage of the interception maneuver, the bat approaches so close to the insect that the delay of echoes is too short for the auditory system to register each echo as a distinct sound arriving just after the broadcast. Nevertheless, the bat orients to the insect and reaches out with its tail membrane to seize the target in midair, exhibiting knowledge of the insect's location in the final moments before capture. How does the bat perceive the insect and distinguish it from other objects during the terminal stage of pursuit if echoes arrive too soon after the broadcasts to directly evoke neural responses on their own? We describe how the bat's auditory system represents echo phase—one of several parameters of echoes related to the target's shape and one that must be perceived to distinguish the insect from other objects. During the terminal stage, even though individual echoes from the insect do not seem to evoke neural discharges distinct from those discharges specifically evoked by the broadcast sounds, the pattern of responses evoked by broadcasts and echoes together encodes the phase of echoes in a stable format throughout the interception maneuver.

Interception of Flying Insects

The pursuit maneuver

Figure 1 shows the interception of a flying insect by the big brown bat, *Eptesicus fuscus*. This common North American bat uses its sonar to locate and track such prey as flying moths, mayflies, or beetles (Kurta and Baker, 1990). The big brown bat usually hunts for insects in open spaces several meters from obstacles such as trees, buildings, or the ground. Under quiet conditions, *Eptesicus* can detect insect-sized targets at maximum ranges of 3–5 m (Kick, 1982), and the bat quickly approaches and captures its prey once detection has occurred. Figure 1 is a composite diagram made from photographic and video studies of individual pursuits in both laboratory and field conditions (Griffin, 1958; Saillant *et al.*, in prep.; Lee *et al.*, 1995). The bat is depicted as being in an open area with the insect initially about 1.5 m away. (Successive images of the bat and the insect are marked from #1 to #7 and show the locations of the bat and the insect at time intervals of about 100 ms.) The bat approaches the insect at a relatively constant velocity of about 3 to 4 m/s (Lee *et al.*, 1995), which leads to completion of the maneuver from Figure 1 in about half a second. During the pursuit, the bat emits a series of sonar sounds to guide its flight, changing these sounds progressively as it approaches nearer to the target. Interceptions

covering target distances from 1.5 m down to capture (as in Fig. 1) typically involve the production of roughly 30–40 separate sonar signals and reception of a corresponding number of echoes. Analysis of the bat's sounds and its behavior during the interception maneuver reveals a great deal about what the bat must perceive from the echoes it receives (Griffin, 1958; Kuc, 1994; Novick, 1977; Schnitzler and Henson, 1980; Simmons *et al.*, 1995a).

Characteristics of sonar sounds during pursuit

Eptesicus broadcasts frequency-modulated (FM) sonar sounds that are relatively brief (Hartley, 1992; Simmons, 1989). Figure 2 shows the bat's sonar broadcasts (solid spectrograms) and the echoes (dashed spectrograms) that the bat's ears would receive during a pursuit similar to that in Figure 1. (The sequence of numbers from #1 to #7 shows the approximate relation between the sounds in Fig. 2 and the numbered images of the bat and the insect in Fig. 1.) The spectrograms making up this "pursuit sequence" illustrate the general pattern of auditory stimulation that occurs during echolocation. (Fig. 2 also illustrates the general pattern of neural responses evoked by FM broadcasts and echoes; see below.) The bat's FM emissions and the echoes they produce contain first and second harmonics that together span the frequency range from roughly 20 kHz to 100 kHz (FM₁ sweeps from 55–60 kHz down to 24 kHz; FM₂ sweeps from 100–105 kHz down to 48 kHz). As the bat flies closer to the insect (Fig. 1), the delay of echoes shortens because the path-length traveled by the sound is reduced, and the bat reacts by shortening its sounds proportionally and increasing the rate of its emissions (Fig. 2).

In Figure 1, the length of the emitted sound as it propagates through the air at each numbered point in the interception (#1–#7) is shown by a grey line from the bat through the insect (34 cm of "sound-length" per millisecond of duration). The first broadcast signal in Figure 2 is about 8 ms in duration (at image #1 in Fig. 1), and succeeding signals shorten progressively from 8 ms down to about 1–2 ms during the approach and tracking stages (image #1 to image #5 in Fig. 1, corresponding to the first two-thirds of Fig. 2). At the beginning of the terminal stage the duration of the broadcasts is about 1 ms, and the duration rapidly shortens to 0.5 ms for the remainder of the flight (image #5–image #7, beginning at arrow alongside bat's flight-path in Fig. 1, corresponding to the latter one-third of Fig. 2), until the bat seizes the prey in its tail membrane (image #7 in Fig. 1). The interval between successive broadcasts also shortens, from about 50 ms at the start of Figures 1 and 2 to about 7 ms at the end, presumably because the bat takes advantage of the

steadily declining echo delay to squeeze in more sounds for more rapid updates of the sonar images (Griffin, 1958; Hartley, 1992; Novick, 1977; Schnitzler and Henson, 1980; see model of interception by Kuc, 1994).

Perception of Targets

Echo delay and target range

Each broadcast sound illuminates, or *ensonifies*, the target for only a few milliseconds, followed by a silent interval during which echoes are received (Fig. 2). During the interval following each broadcast, echoes return from objects at different distances according to the velocity of sound until echoes become so weak that they can no longer be detected (Griffin, 1958; Lawrence and Simmons, 1982). In air, the delay of echoes is 5.8 ms per meter of target range. Because *Eptesicus* has a maximum effective operating range of about 5 m for its sonar system, the bat can be expected to experience delays up to about 30 ms (Kick and Simmons, 1984) and routinely must cope with delays from less than 1 ms to 15–20 ms during its nightly activities (Griffin, 1958). In Figure 2, the first echo has a delay of about 10 ms. Successive echoes decrease in delay as the bat flies nearer to the target until delays become as short as 0.3–0.5 ms

just prior to the moment of capture when the insect is only about 5–10 cm from the bat's mouth. The bat regulates the duration of its broadcasts using knowledge of echo delay from one broadcast to the next; at the moment of capture the bat positions its tail membrane to seize the insect (Schnitzler and Henson, 1980; Simmons, 1989).

The bat perceives the target's direction and distance from the echo received after each broadcast and updates the images from one broadcast to the next to follow the target's changing position. When the insect flies near branches and leaves (several kinds of night-flying insects take such evasive action upon hearing the attacking bat—see Fenton, 1995), the bat must perceive the direction and distance of the vegetation, too, because it chases the insect while also avoiding collisions with these other objects (Simmons *et al.*, 1995a). To be precise, the bat must be able to perceive the locations of multiple targets while at the same time perceiving which target is the insect and which targets are part of the background. During the terminal stage, the bat makes last-minute adjustments in its flight-path and its posture to swing its tail membrane up behind the target, seize the target, and bring it to the mouth. If the insect has flown into vegetation, background objects are avoided as well; pho-

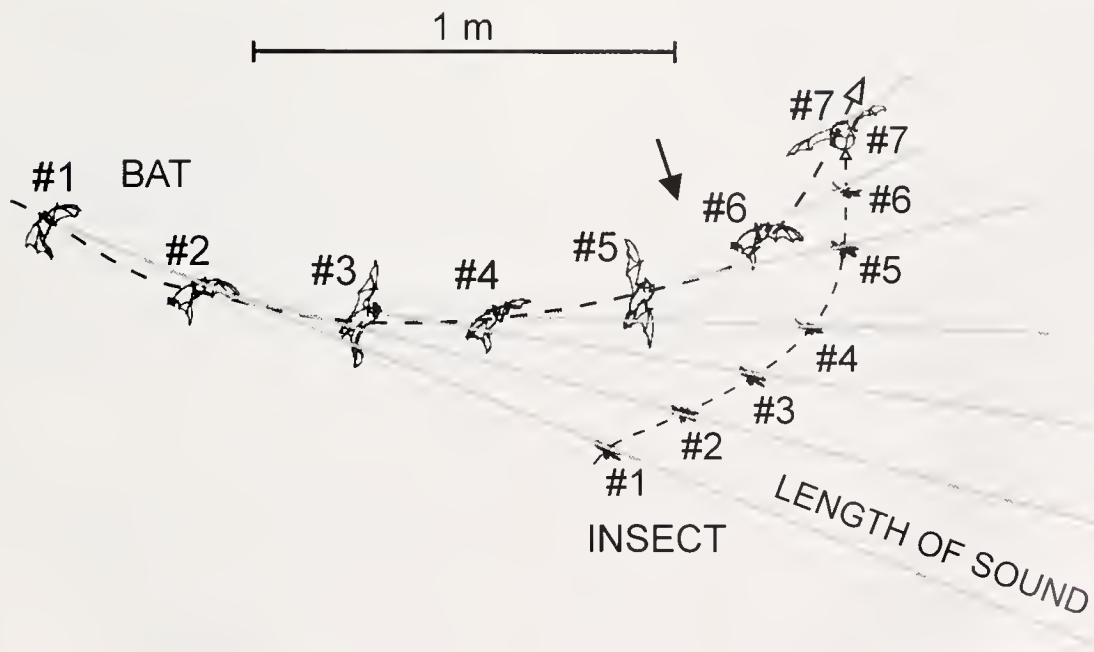


Figure 1. Diagram of an echolocating big brown bat intercepting a flying insect. Images numbered #1–#7 show locations of the bat and the insect at intervals of approximately 100 ms in this composite of numerous flights recorded in different studies. The bat emits sonar sounds throughout the maneuver (see Fig. 2), and the spatial extent of the broadcast sounds at points corresponding to the numbered images is shown by grey lines from the bat through the insect. The arrow near bat image #6 marks the location of the transition to the terminal stage of pursuit and locates the region of interest below.

tographs and video recordings reveal the bat's impressive agility during these maneuvers (see fig. 4.8 in Simmons *et al.*, 1995a). To achieve continuity in perception of the rapidly changing target scene, the bat somehow must carry information about the insect from one image to the next to avoid losing the insect in the background. We represent this continuity by a wide horizontal grey bar (labeled as perceived image) extending along the whole series of emissions and echoes in Figure 2.

Target shape and echo spectra

Insects are not just single reflecting points; they are small objects with dimensions from a few millimeters up to a centimeter or two between their principal body parts (*e.g.*, the head, the wings, and the abdomen). A typical insect therefore returns not just one "echo" but several

reflections—a replica of the incident sonar sound from each prominent body part or *glint* (Kober and Schnitzler, 1990; Moss and Zagaeski, 1994; Simmons and Chen, 1989; Simmons *et al.*, 1995a). In sonar, the target's shape can be represented along the axis of range by the spacing of the glints, or by the time separation of the overlapping reflections, which might be up to about 100 μ s for insects of the sizes commonly pursued by *Eptesicus* (Simmons, 1989). The overlapping reflections interfere with each other to produce reinforcement and cancellation at different frequencies, which modifies the spectrum of the overall echo the bat receives from the insect relative to the spectrum of the sound that was originally transmitted. Interference also modifies the phase of echoes relative to the incident sound, and both the amplitude and the phase of echoes are potential cues for perception of target shape (Simmons, 1989).

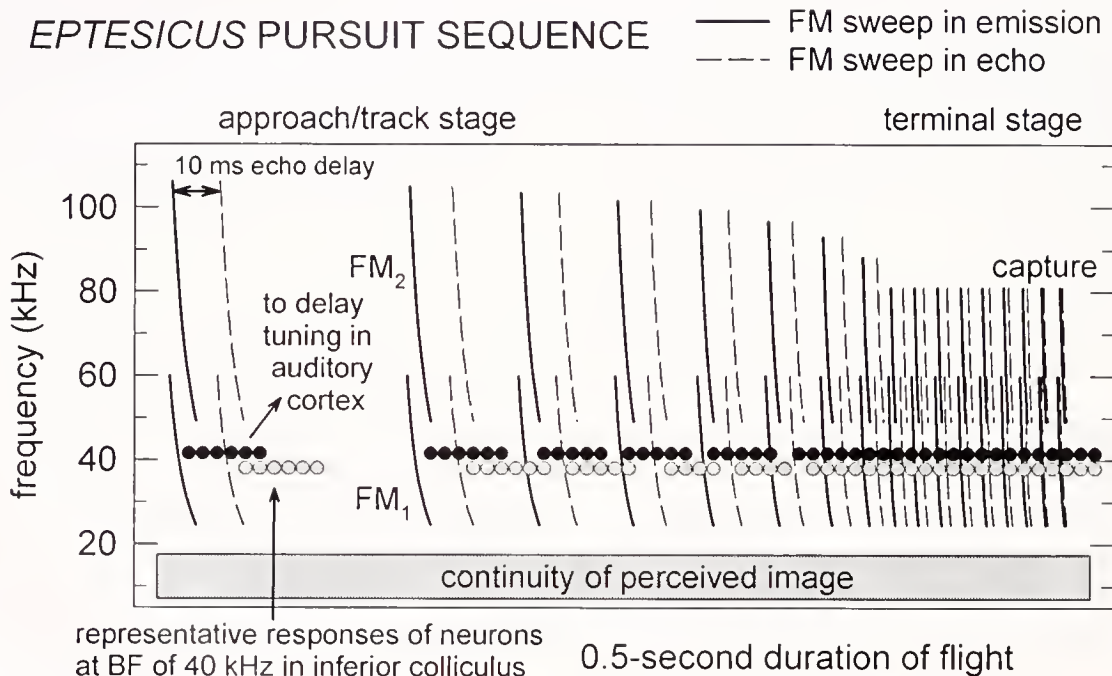


Figure 2. Diagram tracing general features of spectrograms for the bat's FM broadcast sounds (solid) and FM echoes (dashed) received from the insect during the pursuit in Figure 1. The numbers #1-#7 at top show the approximate relation between these sounds and the images of the bat and the insect in Figure 1. The arrow at #6 shows the transition to the terminal stage of pursuit. This diagram also shows the relation between occurrences of a specific frequency of 40 kHz in the FM sweeps and responses of single neurons in the inferior colliculus tuned to a best frequency (BF) of 40 kHz. Black dots show neural responses evoked by the broadcast sounds, and grey dots show responses evoked by echoes; these responses act as inputs to delay-tuned neurons at higher levels of processing (*e.g.*, auditory cortex). In the terminal stage (arrow), responses to the emissions and echoes run together, and the echoes themselves no longer evoke clear-cut responses separate from the emissions; instead, responses are triggered just by the emissions. Latencies of responses in the inferior colliculus are different for different neurons tuned to the same BF (*e.g.*, 40 kHz), and different neurons respond to different emissions or echoes at different points along the maneuver. As a result, the neural representation of the target cannot critically depend on which cells respond, apart from their BF and latency values. Continuity in the bat's perception of the insect throughout the flight is represented by the long horizontal grey bar.

Perception of target shape

Eptesicus perceives changes in the fine delay (glint) composition of echoes as well as their amplitude spectrum and phase (Menne *et al.*, 1989; Mogdans and Schnitzler, 1990; Moss and Schnitzler, 1995; Simmons, 1989; Simmons *et al.*, 1989; 1990a, b; 1995a). For example, in various discrimination tasks, *Eptesicus* readily can detect small changes in the frequency of spectral peaks and notches as well as shifts in echo phase (e.g., 0° to 180°, -90° to +90°, -45° to +45°). The bat's ability to perceive changes in the amplitude spectrum of echoes is not surprising: its auditory system represents ultrasonic frequencies topographically, thus creating a map of the acoustic spectrum in terms of activity levels in neurons tuned to different frequencies (Covey and Casseday, 1995; Kössl and Vater, 1995; Neuweiler, 1990; Pollak and Casseday, 1989). However, the ability to perceive ultrasonic echo phase-shifts is unexpected (Pollak, 1993; Schnitzler *et al.*, 1985); it requires that neural discharges in lower auditory centers exhibit some degree of synchronization to peaks and valleys in the cycles of ultrasonic sounds, and, furthermore, that discharges of individual higher-level neurons somehow preserve this information on a time scale of only a few microseconds. The main problem stems from the variability actually observed in the timing of neural discharges (in the bat's inferior colliculus; see below), which typically is hundreds of microseconds—much larger than the timing precision seemingly needed to record changes in echo phase (Pollak *et al.*, 1977; Schnitzler *et al.*, 1985). Nevertheless, several experiments show that the limit for detection of echo-delay or phase-related alternations in delay by *Eptesicus* is smaller than 0.5 μ s (Moss and Schnitzler, 1995; Simmons *et al.*, 1995a). The minimum threshold seems to be about 10–15 ns (Simmons *et al.*, 1990a). Until recently it has seemed physiologically impossible for bats to represent the phase of echoes, but in fact it is computationally feasible even with the known limitations inherent in neural responses recorded from the bat's auditory system (Saillant *et al.*, 1993; Simmons *et al.*, 1995b). How does the bat represent echo delay or phase so it can detect small changes (in the range of a fraction of a microsecond) in the timing of FM sweeps if the noisiness in neural responses amounts to hundreds of microseconds?

Auditory Representation of Sonar Emissions and Echoes

Transient, distributed character of single-unit responses to FM emissions and echoes

During echolocation, each of the bat's auditory receptors "looks at" the FM sweep in the broadcast sound or

in an echo for only that portion of the sweep that passes near its tuned frequency (Kössl and Vater, 1995). During the late approach and terminal stages of pursuit, the bat's FM signals are only about 0.5–3 ms long, which provides a very short (several hundred microseconds) effective duration for auditory stimulation at each frequency (Simmons *et al.*, 1989). On-responses marking the time of occurrence of successive frequencies in FM sweeps are passed from cell to cell upward along the bat's auditory pathway. These responses travel from the auditory nerve through the cochlear nucleus and other brain-stem sites to the inferior colliculus, which is a major auditory processing center in the bat's brain (Covey and Casseday, 1995; Pollak and Casseday, 1989; Simmons *et al.*, 1995b). The inferior colliculus is a good location in which to examine how responses might encode information about targets that the bat eventually perceives. This auditory processing center gathers responses from all auditory nuclei in the lower brain-stem and imposes some degree of integration before sending information to higher auditory centers and to motor centers (Casseday and Covey, in press; Covey and Casseday, 1995; Pollak and Park, 1995). Analysis of the timing of on-responses in neurons of the inferior colliculus to specific frequencies in FM sweeps indicates that the effective stimulus for echolocation (which is the occurrence of the specific frequency band or segment of the FM sweep that excites each neuron) indeed lasts for only a few hundred microseconds and leads, on the average, to just one spike for each stimulus presentation in each neuron (Bodenhamer and Pollak, 1981; Ferragamo, 1994).

Figure 2 illustrates schematically the timing of neural responses in the inferior colliculus of *Eptesicus* in relation to the FM sweeps in the bat's sonar emissions and the echoes received from a target corresponding to the insect in Figure 1. Each neuron is tuned to a specific frequency ("best" frequency, or BF) between 10 and 100 kHz, and each cell registers the time of occurrence of its best frequency in a short-duration FM sweep with a single spike at a specific latency (Casseday and Covey, 1992; Ferragamo, 1994; Haplea *et al.*, 1994; Jen and Schlegel, 1982; see Covey and Casseday, 1995). The diagram in Figure 2 shows rows of dots representing single-spike responses of individual neurons tuned to a best frequency of about 40 kHz on the vertical frequency axis (black dots for responses to emissions and grey dots for responses to echoes; each dot represents a different neuron). Although these neurons are all tuned to a frequency of 40 kHz, their responses nevertheless differ in latency. At any particular frequency there are neurons in the inferior colliculus with latencies ranging from about 3–4 ms to about 25–30 ms, with a scattering of longer latencies out to 40–50 ms or more. As the bat's pursuit of

the insect continues, the broadcast sounds and echoes come closer together and shorten in duration. Concomitantly in Figure 2, the bursts of responses these sounds evoke also come closer together and eventually merge into a more-or-less continuous series of spikes in different neurons (still one spike per neuron for each broadcast or echo, however). Even in the terminal stage, each broadcast is able to evoke responses in individual neurons of the inferior colliculus because the minimum interval between broadcasts is about 7 ms. Eventually, however, the echoes in the terminal stage arrive too soon to evoke responses separate from those occurring to the broadcast because the delay of echoes falls below the minimum recovery time of about 2 ms for the inferior colliculus. (In the terminal stage, the black dots and the grey dots in Fig. 2 are triggered by the solid spectrograms, with no dots triggered by the dashed spectrograms.) The problem therefore is that neural responses in the latter part of the pursuit are all evoked by the broadcast, none by the echo, while the target itself necessarily is represented only by the echo.

Early in the pursuit maneuver, when the repetition interval between the broadcasts is long (>50 ms between successive sounds) and the delay of echoes is long (>10 ms), neurons in the bat's inferior colliculus are able to respond separately to each emitted sound and then to each echo. This response is possible because the limiting recovery times for the neurons to respond to the second of two transient stimuli are shorter than the intervals between successive sounds and their echoes. However, as the repetition rate of emissions increases and the delay of echoes decreases, the acoustic stimuli encroach on limitations in the rate at which different neurons can follow rapid sequences of sounds or recover their responsiveness in time to respond to the echo as well as the emission (Casseday and Covey, in press; Casseday *et al.*, 1994; Condon *et al.*, 1994; Covey and Casseday, 1995; Haplea *et al.*, 1994; Jen *et al.*, 1993; Moriyama *et al.*, 1994; Pinheiro *et al.*, 1991). Some neurons that might respond to the first emission, to the first echo, and then to the second emission thus will "drop out" when the second echo occurs, to be replaced by other cells with more favorable response characteristics (they prefer faster rates or shorter delays). Consequently, throughout the approach and tracking stages of interception, as echo delay and the interval between emissions both become shorter, different subpopulations of neurons appear and then disappear from the overall active population of cells. The already distributed nature of these responses is carried even further in the terminal stage by the fact that no neurons appear to respond directly to the echoes at all during the terminal stage. Instead, responses that convey information specifically about the echoes are merged or "dis-

tributed" into responses to the emission. How does the bat achieve some continuity in its images throughout pursuit if different neurons are used at different points in the sequence and if echoes also fail to elicit discernible responses of their own during the terminal stage?

Neural display of target range

The bat's auditory system uses the timing of neural responses in the inferior colliculus to create a higher-level representation of echo delay that ultimately displays target range in the midbrain and auditory cortex (Dear and Suga, 1995; Dear *et al.*, 1993a, b; O'Neill, 1995; Suga, 1990; Suga *et al.*, 1995). Broadly spread latencies in the inferior colliculus amount to physiological delays imposed on responses to systematically retard and disperse the registration of each frequency by different neurons. Shorter latencies (*e.g.*, <4–5 ms) probably are due to the time required for responses to travel up to the inferior colliculus along different pathways, but longer latencies (>5 ms) appear to be caused by periods of inhibition with different lengths, which produce the delays themselves, followed by brief bursts of excitation to trigger responses at the end of the inhibitory delay window (Casseday *et al.*, 1994; Saitoh and Suga, 1995). The dispersal of latencies shown in Figure 2 creates physiological delay lines whose delay "taps" correspond to individual neurons responding at their characteristic latencies to the emission (black dots) or to the echo (grey dots). These delayed neural responses leave the inferior colliculus and travel upward to the medial geniculate (auditory thalamus) and auditory cortex, where higher-level neurons are placed to receive inputs from both the emission and the echo. These receiving cells are *coincidence-detectors* that each produce a spike when, and only when, their inputs from the emission and the echo arrive simultaneously (Carr, 1993). By detecting the coincidence, or simultaneity, of its inputs, the coincidence-detector becomes tuned to the time difference between the response latencies to the emission and the echo, or echo delay (O'Neill, 1995; Suga, 1990; Suga *et al.*, 1995). In *Eptesicus*, coincidence-detectors in the auditory cortex are tuned to echo delays from 2–3 ms to 28 ms, with different neurons having different best delays (Dear *et al.*, 1993a, b). The delay-tuning curves of these neurons represent target ranges from about 0.3–0.5 m to about 5 m, which covers most of the operating range of the big brown bat's sonar (Kick, 1982; Kick and Simmons, 1984). The accuracy with which delay-tuning curves represent target range is limited, however: the width of delay tuning in *Eptesicus* is no better than about 0.5–1 ms even in the most sharply tuned neurons and typically is several milliseconds for each cell (Dear *et al.*, 1993a, b).

The bat as a whole has an echo-delay acuity of a micro-second or less (Moss and Schnitzler, 1995). The width of delay-tuning curves in *Eptesicus* appears to be more closely related to the coarse scale of delay acuity associated with discrimination of differences in target range (50–100 μ s; Moss and Schnitzler, 1995; Simmons, 1973) than to the fine delay acuity associated with perception of phase and glints (0.01–1 μ s; Simmons *et al.*, 1990a, b).

Interestingly, the only portion of the bat's sonar operating range not covered by cortical delay-tuned neurons is the short end from about 0.5 ms to 2 ms which the bat encounters during the terminal stage of pursuit, when it has come within about 0.3–0.5 m of the insect. Such short ranges also are encountered when the bat flies to a landing site (see Lee *et al.*, 1995) or examines any objects closer than 0.3–0.5 m. This is the region where neurons in the bat's inferior colliculus can no longer produce separate responses to echoes and thus can deliver no echo inputs to drive delay-tuned coincidence-detectors. Nevertheless, the bat perceives the range to the insect during the terminal stage, including such details about range as are provided by the spectrum and phase of echoes. During the terminal stage, how does the bat represent target range, including the fine delay information implicit in its ability to perceive changes in echo phase as changes in delay? For that matter, how does the bat perceive echoes at all during the terminal stage?

Overcoming the distributed character of the neural representation for targets

On the average, each neuron in the bat's inferior colliculus discharges only once and thus participates in actively representing an echo for only the brief duration of its spike. Other neurons discharging at other times carry the representation in the inferior colliculus over the entire epoch of roughly 30–35 ms during which the echo from the target is processed, which lasts more or less until the next broadcast sound is emitted (Simmons *et al.*, 1995b; see Fig. 2). The same is true for delay-tuned neurons in the bat's auditory cortex; each delay-tuned cell registers an emission-echo coincidence with, on the average, a single spike and thus only very briefly contributes to the representation of echo delay. Other delay-tuned neurons responding at later times carry the representation across the whole epoch of 30–35 ms associated with processing echoes from each broadcast sound (Dear *et al.*, 1993a, b). The distributed character of the representation of targets is not occasioned solely by the occurrence of an average of only one spike in each neuron. Additional distribution occurs because the same neurons do not follow the emission or the echo throughout the entire pursuit. Both in the inferior colliculus and in the

auditory cortex, different neurons will respond to emission-echo pairs at different stages of the pursuit maneuver because these cells differ in their preferences for the duration, repetition rate, and other temporal features of the train of stimuli. Toward the end of the flight, responses to echoes are distributed even further in the sense that no obvious responses are triggered at all by echoes in the terminal stage. This is the essence of the distributed character of the bat's auditory code for echo delay. To achieve some degree of continuity in its images of the target, the bat must have a means for integrating responses across multiple neurons to overcome the distributed character of the neural representation for each target. Recordings made in the inferior colliculus from local groups of neurons discharging together for each stimulus provide a means to bypass the dispersion of responses observed one cell at a time and achieve the desired integration of information about the target in actual experiments (see Simmons *et al.*, 1995b). These multiunit responses, recorded directly from neighboring cells in a small volume of tissue, show how groups of cells share the work of representing emissions and echoes.

Multiunit responses in the bat's inferior colliculus

Figure 3 illustrates a series of local multiunit responses recorded with a high-impedance single-unit electrode positioned "between cells"—that is, without having spikes from a single neuron dominate the signal. Each time a stimulus is delivered to the bat's ears, the electrode picks up clusters of spikes mixed together at similar amplitudes from different cells rather than the spikes of just one cell at a very high amplitude. The waveform being recorded is an analog signal made up from a combination of spikes in different cells; the stimulus is repeated numerous times and the response is averaged across these multiple presentations to reveal the presence of synchrony between any of the spikes and the stimulus. Each peak in the response trace in Figure 3 thus shows the simultaneous occurrence of spikes in a group of cells at a fixed latency following the stimulus, and different peaks represent groups of neurons responding synchronously at different latencies. The responses shown in Figure 3 were evoked by presenting the bat with a series of 54 electronically simulated sonar emissions and echoes that mimic a whole pursuit sequence, and then averaging the set of analog responses over 64 independent occurrences of the entire sequence of sounds. Note that these sounds only *simulate* 54 emissions and echoes because the bat is not actually vocalizing the broadcasts; instead, the bat's ears receive stimulation that corresponds roughly to what they would receive if the bat did produce these sonar sounds while approaching a target. The spe-

A IC MULTI-UNIT RESPONSE TO PURSUIT SEQUENCE

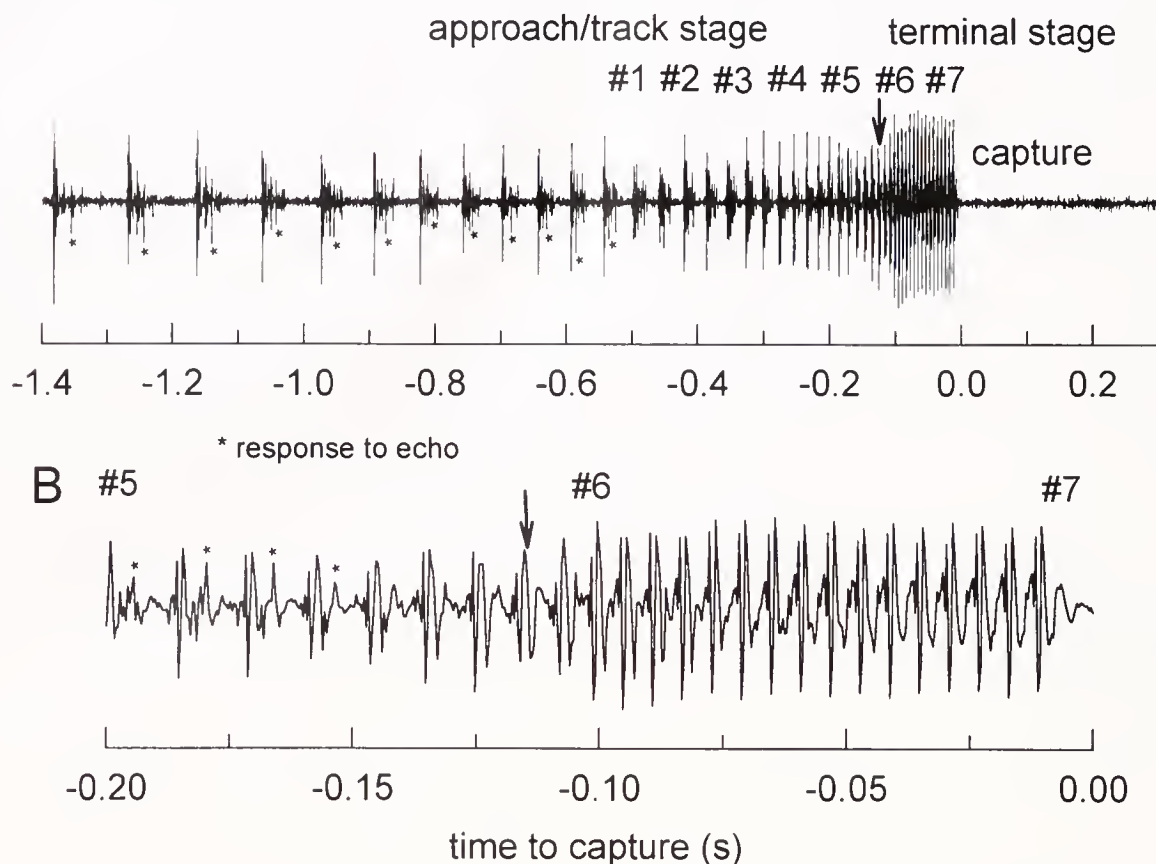


Figure 3. Multiunit responses recorded in the inferior colliculus of *Eptesicus* for a series of acoustic stimuli simulating an entire pursuit sequence of emissions and echoes (whole sequence in A; final 0.2 second of sequence in B). The segment of this stimulus/response sequence that corresponds to Figure 1 and 2 is shown by #1–#7 for the numbered images in Figure 1. The arrow marks the transition into the terminal stage. Asterisks show approximate locations of responses just to the echoes at those points where they are visible in the trace (first 12 visible at scale of A; first 33 visible at scale of B); at other points responses to echoes are either obscured by multi-peaked structure of responses to emissions (at -0.5 s to -0.15 s in A) or else specific responses to echoes are absent due to neural recovery times being slower than echo delay (at -0.15 s to 0 in B, which includes the terminal stage).

cific segment of the artificial pursuit sequence used to generate the responses in Figure 3 that corresponds to the maneuver shown in Figures 1 and 2 is marked with numbers from #1 to #7; these give the approximate locations of the numbered images of the bat and the insect in Figure 1 alongside the multiunit response trace.

In detail, the series of averaged multiunit responses shown in Figure 3 consists of bursts of peaks triggered by each broadcast and its associated echo, with each burst lasting for up to about 30–40 ms. Usually there are “silent intervals” between bursts of responses when no sounds are occurring. However, as the repetition rate of the artificial broadcasts increases, the silent interval be-

tween bursts shortens until, about at the arrow in Figure 3, the bursts of responses run together to form a continuous sequence of peaks. The response trace in Figure 3 contains well-defined peaks that register the reception of each of the 54 simulated sonar broadcasts, followed by several lower-amplitude peaks that correspond, first, to the wide spread of response latencies seen in the inferior colliculus for the emitted sound and, second, to responses for the simulated echoes as well as the broadcasts. In Figure 3, virtually every simulated broadcast produces a response (beginning with the largest peak in each burst of peaks), and most of the echoes produce a response, too (echo responses that are readily visible in

the traces shown in Figure 3A–B have been marked with *). The presence of distinguishable responses to the broadcast and then to the echo is maintained up to the transition between the approach or tracking stages of pursuit and the terminal stage in the artificial pursuit sequence (at arrow in Fig. 3B).

Although the responses in Figure 3 are composed chiefly of spikes that emerge from the averaging process due to their synchronization with the stimulus at different latencies, individual response peaks are not derived from the same neurons at all positions in the sequence. Different neurons contribute to the multiunit response peaks at different stages in the sequence because neurons in the bat's inferior colliculus vary in their preferences for duration, repetition rate, and echo delay. Interestingly, the total number of neurons and level of synchrony making up the responses to the individual emissions appear relatively stable across different emission-echo pairs in Fig. 3A–B because the peaks are roughly of the same height (amplitude variability is less than 50%) and width throughout the whole sequence. Separate peaks occur in response to the echoes (*) for about the first 33 emission-echo pairs in Figure 3, but these echo responses are more difficult to discern because they are lower in height and because they also are mixed with multiple peaks in the response to the emission alone. These multiple peaks correspond to the spread of response latencies for different neurons tuned to the same frequency (e.g., at 40 kHz in Fig. 2). During the terminal stage (starting at arrow in Fig. 3), the disappearance of any obviously separate response peak to each echo is virtually guaranteed because hardly any neurons in the bat's inferior colliculus have recovery times shorter than 1–2 ms. Thus, the general appearance of multiunit responses in the bat's inferior colliculus to simulated sonar broadcasts and echoes appears largely predictable from the results of single-unit studies.

Representation of delay and phase in multiunit responses

The multiunit responses in Figure 3 are analog waveforms obtained by averaging spikes from clusters of neurons over multiple presentations of the artificial pursuit sequence. Individual neurons will either appear in the averages or drop out according to each cell's sensitivity to frequency, amplitude, duration, delay, and repetition rate. Consequently, there is no straightforward way to interpret each multiunit response peak in relation to responses of individual neurons to these stimulus parameters if that is the goal. But what if the bat's brain is not just trying to assemble a rate-coded feature-by-feature description of emissions and echoes during pursuit?

What if the bat's brain instead seeks to reconstruct aspects of the acoustic signals as *time-series* events, perhaps even with scant regard for which neurons produce the responses that make them up (Simmons, 1996; Simmons and Dear, 1991)?

Averaged multiunit responses are not only averaged synchronized spikes from multiple neurons, they are also time-series signals with their own properties of amplitude, peak height, and phase. The neurons whose spikes make up different peaks in the averaged waveform in Figure 3 may respond to only a portion of the pursuit sequence—perhaps to a few of the emissions or the echoes out of the whole series of emission-echo pairs, but the parameters of the multiunit responses appear not to depend critically on this fact because all the emissions and many of the echoes evoke specific peaks in these responses. What would happen to the multiunit responses *as signals* if echo delay or phase were changed by a few microseconds? Figure 4 illustrates just one example of the surprising dependence of local averaged multiunit responses from the inferior colliculus of *Eptesicus* on fine temporal features of emissions and echoes (Simmons *et al.*, 1995b). The figure shows the positive-negative-positive series of peaks in the multiunit response evoked at a latency of 4.4–5.2 ms by one of the emissions taking place early in the terminal stage (at arrow in Fig. 3), when the delay of echoes is about 1 ms—too short for the echo on its own to evoke strong responses in the inferior colliculus. (The horizontal time scale in Fig. 4 is expanded relative to Fig. 3 and has its origin shifted to the onset of the specific emission-echo pair #37 designated by the arrow in Fig. 3. Consequently, time values in Fig. 4 are latencies relative to the artificial emitted sound.)

All the emissions and the echoes used to produce the responses shown in Figure 3 are set to be *normal in phase* relative to each other and at the same time *inverted in phase* relative to an arbitrary, external phase standard (180° phase, or I,I in the terms used in the legend for Fig. 4). Figure 4 shows what happens to a representative peak from Figure 3 when the phase of the emitted sound or the echo is altered from this initial 180°/180° (I,I) condition. (The solid line in Fig. 4 is labeled I,I for “inverted” or 180°/180° phase of the emission and the echo together.) The five curves in Figure 4 then show different phase conditions for the emission and the echo (I,I for 180°/180°; N,N for 0°/0°; I,N for 180°/0°; N,I for 0°/180°; and I,I for a repeat of 180°/180°). The chief effect of these changes in phase for the emission and echo is that the response peaks in Figure 4 slide sideways over a time interval of up to several hundred microseconds according to the phase of the emission in relation to the echo. This effect is not restricted to the particular response peak selected for Figure

Response to terminal-stage emission-echo pair for different combinations of emission and echo phase

N = normal (0°) phase, I = inverted (180°) phase

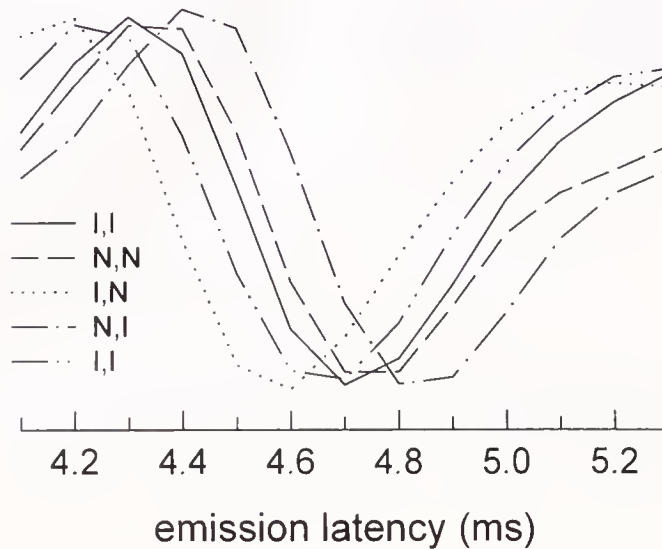


Figure 4. Waveform of the peaks in the multiunit response marked by the arrow in Figure 3B (see also arrows in Figs. 1 and 2). The first response peak shown here has a latency of about 4.4 ms and originates from within the inferior colliculus; nominally it is a response to an emission from the terminal stage, when responses specifically associated with the echoes no longer are visible by themselves. Although there is no clear response peak associated with the echo at this stage, the latency of the illustrated response peak for the emission nevertheless shifts according to the phase of both the emission and the echo ($N = 0^\circ$; $I = 180^\circ$; see text). The responses to the emissions in Figure 3, at latencies of 4–5 ms after each emission, all show large latency shifts of 100–200 μ s associated with 0° and 180° phase shifts in these simulated emissions and echoes. Evidently the responses to each “emission” in fact are responses to a complex of sounds that must include at the very least the previous echo along with the current emission. The phase of the echo is represented consistently throughout the pursuit maneuver, but this information resides in an unexpected place—in the latencies of peaks in what would nominally be considered the response to the emission.

4; all of the peaks in the multiunit responses evoked by the emissions in Figure 3 slide the same way with changes in emission or echo phase as shown in Figure 4. This effect can only mean that latencies of the neural spikes making up the peaks in the multiunit responses to the emissions have somehow become consistently dependent on the phase of the original ultrasonic emissions, echoes, or both. The magnitude of the latency shifts associated with the 180° phase shifts is especially interesting: For the stimulus, a phase shift of 180° in the ultrasonic signal moves the cycle-by-cycle structure of the acoustic waveform by only about 5 to 20 μ s (half the ultrasonic period) depending on the frequency at each point in the FM sweeps, yet for the responses, the peaks in Figure 4 slide in latency by 50–200 μ s.

Magnification of microsecond stimulus time scale in multiunit neural responses

Two significant aspects of the phase-shift effect in multiunit responses from the bat's inferior colliculus deserve comment. First, the size of the latency shift in the response is as much as 100–200 μ s for some combinations of emission and echo phase. This is roughly 10 times greater than the changes in timing for the cycles of the sound associated with the phase shifts. For this effect to appear at higher auditory processing centers (*e.g.*, inferior colliculus), there must be some mechanism for magnifying or expanding small differences that occur on a scale of a few microseconds in the timing of neural responses at lower auditory processing centers. In the infe-

rior colliculus, the time-scale magnification is roughly a factor of 10 for $0^\circ/180^\circ$ phase changes in emissions or echoes. We have observed time-scale magnifications of roughly 10–100 for various other delay changes in echoes (Ferragamo, 1994; Simmons *et al.*, 1995b). This phenomenon appears robust and ubiquitous in the inferior colliculus and auditory cortex of *Eptesicus*. Moreover, for a given recording site, the same size of time magnification affects all the responses in the pursuit sequence in the same manner, providing a stable, consistent representation of the detailed structure of emissions and echoes without requiring any individual neurons to respond consistently. The locally recorded analog multiunit response thus carries information necessary to explain the bat's performance without this information manifesting itself in conventional single-unit recordings.

The second significant aspect of the phase-shift effect is that the magnitude and direction of the latency shifts depend not only upon the current stimulus phase conditions but also on the previous phase conditions. Notice that the latencies of response peaks in Figure 4 for the second occurrence of the I,I or $180^\circ/180^\circ$ phase conditions do not match latencies for the first occurrence of this condition. That is, when the original (I,I) stimulus condition is restored, the multiunit responses do not shift back to their original latencies; instead, they are offset, in this example by about $40 \mu\text{s}$. We have observed many similar examples of latency offsets in responses to phase shifts and other stimulus parameters that appear to depend on the stimulus conditions previously presented (Simmons *et al.*, 1995b). In general, the time-scale magnification of latencies does not register just the absolute phase of emissions or echoes by itself (*e.g.*, 0° or 180° for either sound) but instead registers their relative phase coupled with a reference latency that depends upon the stimulus conditions that occurred previously.

It thus seems as though at least part of the basis for continuity in the neural representation of echo phase, and therefore perception of target shape, across the pursuit sequence may reside in the latencies of responses to emissions distributed across multiple neurons on a magnified time scale. The apparent absence of responses to echoes in the terminal stage (due to neural recovery times that are too short) may not be a problem for the bat, because information about echoes (*e.g.*, echo phase) actually resides in responses nominally associated with the emissions, not with echoes. The relatively large variability of latencies for individual neurons in the inferior colliculus (which typically is in the range of hundreds of microseconds and which should obscure the presence of the much smaller time shifts of a few microseconds associated with stimulus phase) also may not be a problem for microsecond-scale perception of echo delay. By plac-

ing fine timing information in response latencies on stretched time scales, the bat overcomes the problem. In effect, the bat can "read" echo delay and phase information from neural responses as though the observed latency variability were reduced by the time-magnification factor associated with the responses. The observed latency variability of hundreds of microseconds thus may be only a few microseconds as far as the bat is concerned. Because all the multiunit responses to the emission in Figure 3 exhibit a time shift of the same size (Fig. 4) in spite of the failure of the same neurons to respond consistently across all the sounds in the sequence, these responses call into question the whole idea that rate-tuned responses of single units are the minimal computational elements used by the brain to create images for perception (Simmons, 1996). Instead, vital aspects of perception appear to be manifested in the timing of responses irrespective of which neurons are doing the responding. We have observed a whole family of similar magnified multiunit response-latency shifts caused by changes in the phase of the emission or the echo, the interaural delay difference of the echo, and the time separation of overlapping reflections from target glints (see Simmons *et al.*, 1995b). In each case, the latency of peaks in the multiunit response changes by a larger amount than the stimulus—typically 10 to 100 times greater. The use of multiple time scales in neural responses is itself a novel observation that raises questions about how to interpret responses in relation to representation of stimuli.

Acknowledgments

This research was supported by ONR Grant Nos. N00014-89-J-3055 and N00014-95-1-1123, NIMH Grant No. MH00521 (RSDA), NSF Grant No. BCS-9216718, NIMH Training Grant No. MH19118, McDonnell-Pew Grant No. T89-01245-023, and DRF funds.

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Sensory Ecology: Introduction

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Sensory ecology is a newly emerging integration of two well-established disciplines that had not been examined jointly until Dusenbery's recent book (1992). Although biologists have always recognized the important interrelationships between sensory physiology and ecology, this work was the first to explicitly examine the two together. The brief discussion of sensory ecology with which we introduce the next two papers is taken entirely from the first three chapters of Dusenbery's book. We hope we've done him justice.

Ecology usually traces the flow of energy throughout a system, but in sensory ecology information is the coin of the realm. Thus Dusenbery defines sensory ecology as addressing two major questions: (a) what information is available in the environment and how is it transmitted to the animal; and (b) how do animals acquire and use that information. In the present context it makes most sense to discuss these in reverse order.

Organisms need information to solve at least three kinds of problems: (a) to maintain an appropriate environment, *i.e.*, homeostasis; (b) to time activities (*e.g.*, seasonal changes in behavior) or synchronize activities with those of conspecifics; and (c) to locate and respond to resources or threats (*e.g.*, by moving towards resources or evading or attacking threats). Organisms also need to transmit information in order to influence another's behavior: to identify themselves, warn conspecifics of danger, coordinate activities, or deceive.

Information is passed from a source, by way of a channel, to a receiver. In sensory ecology the source is the environment, which may also be another organism; the channel is a stimulant, for example light, sound or chem-

icals; and the receiver is the organism. Informational interactions between organisms are commonly classified according to whether the interaction has some adaptive advantage to the transmitter, the receiver, neither, or both. In true communication the source emits an adaptive signal that is also adaptive for the receiver. In deception the source emits a signal that is adaptive for the source but maladaptive for the recipient.

An organism's use of information depends upon what information is available in the environment. Information therefore must be carefully defined, and Dusenbery uses information theory as an aid in this definition. Information theory focuses on the maximum amount of information that can be transmitted in a given situation, but says nothing about its content. However, the behavior of an organism depends upon both amount and content, and so consideration must expand to include both. The measurement of information content is a science in its infancy. Information has been defined as the capacity to organize a system, and information transmission as the removal of uncertainty. Thus, a measure of information content is the amount of uncertainty that has been removed.

Careful consideration of stimulus dimensions indicates that the environment contains an infinitude of information, far more than any organism can handle. "The major focus of sensory ecology is thus to ask precisely what information is used by various organisms."

Vince Dethier was delighted with *Sensory Ecology* when it appeared; in part because it formalized one avenue of approach he took. He asked how much of the information in a specific chemosensory signal was available to flies and how much of that information did they use (Smith *et al.*, 1983; Dethier and Bowdan, 1988). It was therefore wholly appropriate, both to the intellectual aims of this symposium and to Vince's spirit, that this topic be addressed.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

In this symposium Dusenbery (1996) examines the information that might be available to a nematode and develops models to explore how that information might be adaptive for this tiny animal.

Atema (1996) also examines information that is available to his favorite organism (the lobster) and asks how that information might direct the lobster towards its resource. Like Dethier, Atema is most interested in the chemosenses, but he concentrates more on olfaction than taste. He has used colored plumes to show that chemosensory information is distributed in complex eddies by nonlinear flows. Thus lobsters must decode complex spatial and temporal patterns. Like Dethier, Atema asks how much of the available chemosensory information the animal can perceive, how the peripheral and central

nervous systems process temporal-spatial signals, and how the signals steer and constrain behavior.

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Information Is Where You Find It

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Abstract. A basic problem of foraging is how to obtain information about the location and quality of food. Relevant information is obtained from immediate stimulation, from previous sensory experience stored in memory, and through the genes. Apparently, even the simplest organisms can use information from all these sources. A good example comes from infective juveniles of the plant-parasitic nematode *Meloidogyne incognita*, which only feed after they locate the root of a suitable host plant. Available evidence indicates that carbon dioxide from host roots attracts the juveniles but that it would also be useful for the juveniles to move to an optimal soil depth in which to search for chemical gradients. Although there are few cues to soil depth, *M. incognita* is able to solve this problem in a surprising way. Juveniles can orient to extremely shallow temperature gradients that are nearly always present in the environment. The juveniles also have the peculiar property of moving toward a preferred temperature that is set several degrees above the temperature to which they are acclimated. Computer modeling of this behavior demonstrates that it provides a mechanism for moving to a particular soil depth. Although average temperature is the same at all depths, the amplitude of daily temperature variation declines with depth and provides the required information, which is extracted by interactions among environmental temperature, preferred temperature, rate of acclimation, rate of locomotion, and temperature range of locomotion. Pseudoplasmodia of the slime mold *Dictyostelium* have similar capabilities. This behavior resembles true navigation in that the stimulus used for guidance has no connection to the goal and suggests that even simple or-

ganisms can extract useful information from surprisingly complex stimulus patterns.

Introduction

In foraging for food, a basic problem that animals face is how to make good decisions about finding food and accessing its quality. Plants and microorganisms face analogous problems. Plants compete for sunlight and make choices about how tall to grow based on information about competing plants nearby (Bradburne *et al.*, 1989; Ballaré *et al.*, 1990). Heterotrophic bacteria and protozoa frequently swim up gradients of nutrients to obtain higher concentrations (Armitage and Lackie, 1990). Fungal hyphae grow up similar gradients (Griffin, 1994, pp. 150–151). Photosynthetic microbes swim toward optimal light intensities (Nultsch and Häder, 1988). I will argue that probably all organisms extract and use information in their environment, sometimes in surprising ways.

These behavioral decisions are invariably based on several kinds of information about the environment. By information, I mean simply an agent (energy or chemical) that is important *only* because it is often associated with states of the environment that have inherent importance to the organism (Fig. 1). In other words, all the kinds of agents that act on an organism can be grouped into causal agents that are inherently important because of the chemical or physical effects that they exert and informational agents that are important only because of their association with some causal agent. For example, a chemical carries information if it affects the organism without providing nutritional needs or having toxic effects. A fundamental property of information is that it can easily be ignored by an organism, and special mechanisms are required to capture and store information. As a corollary, information is usually carried by lower

Received 30 November 1995; accepted 2 February 1996.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.

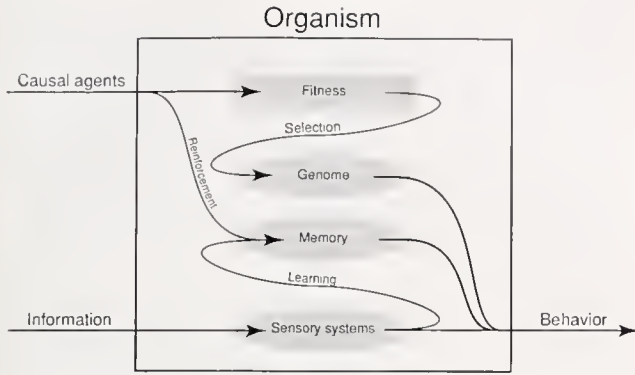


Figure 1. Diagram of relationships between an organism and its environment. On the left are the two types of influences on an organism, and on the right its impact on the environment is represented by its behavior. Shaded ovals indicate the types of information within the organism.

concentrations of chemicals or weaker physical forces than those that directly affect an organism. The scent and color of a flower have importance to a bee only because they are associated with nectar (or pollen), which has nutritional value to the bee. Thus, the scent and color carry information about the presence of nectar or pollen.

As indicated in Figure 1, behavioral activities are regulated by information from three distinct sources: genome, memory, and sensations. The process of selective reproduction results in a store of information in the genome about what decisions and behaviors have been successful in the past for an organism's ancestors. The process of learning results in a store of information in memory about what behaviors have been productive for the individual organism previously. The senses provide information about the current state of the environment. Each of these sources of information thus works over a different time scale and is complementary to the others. I propose that all organisms probably make use of all three types of information, when learning and memory are defined functionally, which leads to the inclusion of sensory adaptation, acclimatization, and habituation (Dusenbery, in press).

One of the most fascinating questions in biology is this: what do organisms know about their environment? In other words, what information do they obtain? This question is difficult to answer for animals other than ourselves and becomes increasingly difficult as organisms differ more from us. A revelation of this century is that some animals can extract information that humans cannot. The ultraviolet light capabilities of insects and the echolocation abilities of bats and whales are clear examples. But what about plants and microorganisms? As we look at simpler organisms, we expect to find more lim-

ited use of information. But how limited? And what information do they obtain? These questions have not been studied very extensively.

In this paper, I present an example of surprising abilities to extract useful information from the complex pattern of temperature changes found in soil in temperate environments by such seemingly simple organisms as nematodes and slime molds.

Patterns of Temperature Change in Soil

Sunlight falling on land surfaces causes relatively large changes in temperature. Daily changes of tens of degrees in the air and soil close to the surface are common as the soil is heated during the day and radiates heat away to the sky at night. Heat travels through soil primarily by conduction and, in most cases, the flow of heat and resulting temperature can be accurately estimated by assuming conduction of heat through a uniform solid (Campbell, 1977). For simplicity, one assumes that the heating and cooling at the surface follows a sinusoidal pattern. Even though the surface temperature pattern is not really sinusoidal, at increasing depths higher frequency components damp out faster than lower frequency components, and a sinusoidal variation with the appropriate fundamental period is a good approximation.

With these assumptions, the temperature at a depth z and time t is described by the equation (Dusenbery, 1992a, p. 115)

$$T(z,t) = T_{\text{ave}} + T_{1/2} \exp\left(-\frac{z}{z_d}\right) \sin\left(2\pi \frac{t}{p} - \frac{z}{z_d}\right)$$

where

T_{ave} is the average temperature at all depths,

$T_{1/2}$ is half the amplitude of the temperature variation at the surface,

p is the period of the variation,

z_d is called the damping depth and equals the square root of the period times the thermal diffusivity of the soil divided by pi ($\sqrt{pD/\pi}$).

For daily variations in temperate zones of the earth, typical values are $z_d = 10$ cm, and $T_{1/2} = 15^\circ\text{C}$. The wavelength of the temperature wave is about 70 cm, and the speed of penetration about 3 cm/h. For annual variations, $z_d = 200$ cm. Within 30 cm of the surface, the temperature gradients are determined primarily by daily variations. From 40 cm to a few meters, annual variations dominate. And temperatures at depths of hundreds to thousands of meters reflect changes in climate.

This temperature pattern establishes vertically oriented temperature gradients, which can guide organisms

upward or downward. At any given depth, warmer temperatures are upward half the day and downward otherwise, but the timing of the change is delayed and the amplitude of the gradients declines with increasing depth. How large are the gradients? The flow of heat from the earth's interior generates an average temperature gradient of $6 \times 10^{-4} \text{ }^{\circ}\text{C}/\text{cm}$. Much larger gradients are usually present near the surface, with $1^{\circ}\text{C}/\text{cm}$ being common in the top few centimeters (Dusenbery, 1988a).

Root-Knot Nematodes, an Example

Nematodes are among the simplest animals with a centralized nervous system. Those that have been studied have a total of only 200 to 300 neurons (Stretton *et al.*, 1978; Chalfie and White, 1988). The root-knot nematodes (*Meloidogyne*) are parasites of plant roots. The spherical adult female develops at a site in a plant root that she never leaves, and she releases eggs to the exterior of the root (de Guiran and Ritter, 1979). The eggs hatch into vermiform second-stage juveniles. These microscopic worms do not feed and must locate an appropriate root before their energy reserves are depleted (in a few weeks during summer). Consequently, the infective juveniles are good models for studying behavioral mechanisms because it is clear that their goal is to move to an appropriate root.

Our experimental studies utilized the southern root-knot nematode (*Meloidogyne incognita*) collected from tomato plants (Diez and Dusenbery, 1989b). Uninfected tomato plants were used as a source of chemical stimuli. Several aspects of nematode behavior were studied by computer tracking methods that recorded the average rate of locomotion of about 200 individuals every few seconds (Dusenbery, 1992b). More traditional methods of observing their net migration on agar surfaces after a period of hours were also used (Diez and Dusenbery, 1989b).

In an extensive search for chemicals emanating from host plants that *M. incognita* infective juveniles might use to locate appropriate roots, we found attraction to carbon dioxide (Pline and Dusenbery, 1987; McCallum and Dusenbery, 1992) but not to any other chemical, although there were also some repellent chemicals released by tomato plants (Diez and Dusenbery, 1989b).

Many root-parasitic nematodes have been found to respond to very shallow temperature gradients (El-Sherif and Mai, 1969), and *M. incognita* does as well, but more detailed experiments revealed that the response was complex. *M. incognita* juveniles moved toward a preferred temperature from both higher and lower temperatures (Diez and Dusenbery, 1989a). This preferred temperature was shifted during acclimation to a different

ambient temperature but was always several degrees above the acclimating temperature. In an appropriate temperature range, they responded to gradients as shallow as $0.001^{\circ}\text{C}/\text{cm}$ (Pline *et al.*, 1988) and showed behavioral responses to temperature changes estimated to be less than 0.001°C (Dusenbery, 1988c). Thus, in the natural environment, they are nearly always in a temperature gradient large enough to influence their locomotion.

What could be the function of such a sensitive but complex response? Due to the dynamic pattern of temperature change in soil near the surface, it was difficult to predict how the nematodes would move, and I turned to computer modeling to address the question (Dusenbery, 1989).

Initial models indicated that estimates of the rate of thermal acclimation and of the width of the temperature range over which the nematodes were active were also needed. More precise data on these parameters were available for the nematode *Caenorhabditis elegans* (Dusenbery *et al.*, 1978; Dusenbery and Barr, 1980), and these values were used as the best available estimates of what *M. incognita* would do. Thermal acclimation occurred with a half time of 2 h. and the temperature range of activity was about 12°C .

As shown in Figure 2, the computer model predicted that during a daily temperature cycle the nematodes would move upward during one or two intervals during the day and downward at other times (Dusenbery, 1989).

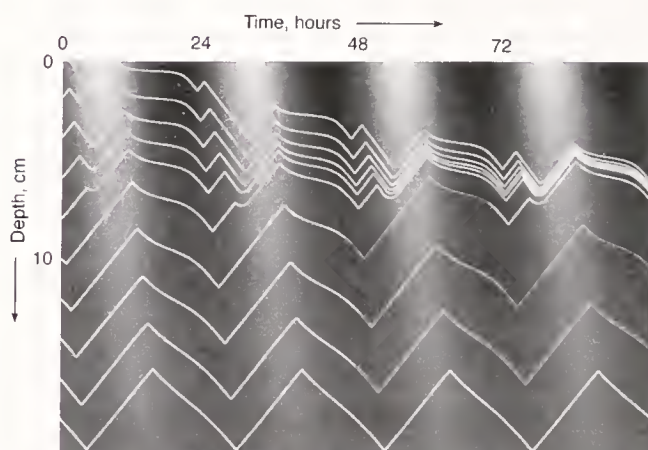


Figure 2. Computed tracks of an organism with the properties estimated for the southern root-knot nematode, *Meloidogyne incognita*. The gray shading represents temperature, with the lighter shades corresponding to higher temperatures. The soil surface is at the top and a depth of 42 cm at the bottom. Time increases to the right over a period of 4.2 days. The white lines record positions of the organisms through time. The various tracks are for organisms starting at depths differing by 5 cm.

However, the distances moved usually did not balance, and the nematodes gradually drifted upward or downward. Most interestingly, nematodes starting out within the top 15 cm of soil all drifted toward a depth of about 5 cm and continued indefinitely to move within a small range at this depth. This "preferred" depth may coincide with a presumed optimal depth at which to search for chemical gradients leading to an appropriate plant root. These modeling results suggest that the thermotactic behavior of these nematodes serves the function of guiding them to an appropriate soil depth and demonstrates that this behavior can extract useful depth information from the complex and dynamic pattern of temperature change.

Nematodes starting out deeper than 15 cm gradually drifted downward until the temperature gradient due to daily fluctuations decays below gradients due to other causes. For example, the average gradient magnitudes of the daily and annual periods are equal at a depth of 30–40 cm (Dusenbery, 1988a; Dusenbery, 1992a, p. 116). It is supposed that relatively few nematodes hatch at these greater depths and that this constant downward movement is a nonfunctional side effect of the mechanism that guides most nematodes toward the optimal depth for finding roots.

Tests of the sensitivity of this behavior to changes in values of the model's parameters demonstrated that the same general pattern of behavior occurs for a wide variety of values (Dusenbery, 1989). However, the rate of locomotion had to be less than 1 cm/h, the temperature range of activity less than 16°C, and the offset of the preferred temperature from the acclimation temperature within the range of 3–5°C.

Although measurement of nematode movement in soil is difficult, Forest Robinson has obtained experimental evidence supporting the hypothesis that *M. incognita* does migrate under the guidance of natural patterns of temperature fluctuation (Robinson, 1994).

Slime Molds, an Example

The other organism that has been demonstrated to have comparable thermal sensitivity is the slime mold *Dictyostelium discoideum* (Bonner *et al.*, 1950; Poff and Skokut, 1977; Whitaker and Poff, 1980). Unlike juveniles of *M. incognita*, pseudoplasmodia of *D. discoideum* move away from a temperature about 2°C below its acclimation temperature. As shown in Figure 3, computer modeling of this behavior, using the best available estimates of the appropriate values of the parameters, demonstrates that pseudoplasmodia starting out within 20 cm of the surface move toward the surface (Dusenbery, 1988b). This movement is even more clearly func-

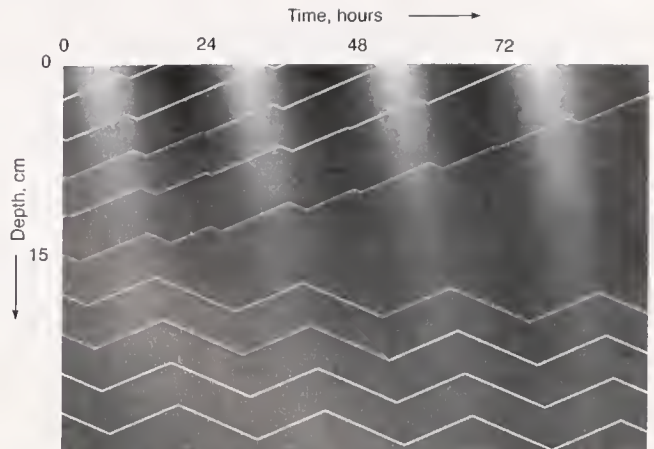


Figure 3. Computed tracks of an organism with the properties estimated for the pseudoplasmodia of the slime mold, *Dictyostelium discoideum*. The parameters are 0.2 cm/h rate of locomotion, 0.2 /h rate of temperature adaptation, -2°C offset of the avoided temperature from the adapted temperature, and an unlimited temperature range of locomotion. Other features as in Figure 2.

tional than the nematode movement. After a period of migration, the pseudoplasmodia form a fruiting body and release spores from the top of a stalk. The spores will be dispersed much more effectively if released at the surface of the soil where wind can carry them. Thus, it appears that even the "simple" slime mold has developed a mechanism for extracting information about depth from complex patterns of temperature change.

Conclusion

In both examples we see a surprising ability of a simple organism to extract and use information from a complex stimulus pattern. In both cases, the behavior is analogous to navigation, because the stimulus used to guide locomotion is not connected to the goal. Such subtle mechanisms may have evolved because alternative stimuli with a simpler relationship to soil depth are lacking. Similar mechanisms could be used by any organism that needs to move with respect to a surface that is subjected to regular heating and cooling (for example, organisms living in a tree trunk). These findings should cause us to look for other situations in which organisms find information where we would not expect it.

Acknowledgments

I appreciate the efforts of two anonymous reviewers whose comments led to significant improvements in the manuscript.

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Eddy Chemotaxis and Odor Landscapes: Exploration of Nature With Animal Sensors

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Introduction

As Von Uexküll, Von Frisch, Tinbergen, and Lorenz before him, Dethier developed methods to study animals from the animal's point of view: He wanted to know both *how* animals perceive their natural environment, and *why* animals perceive their environment in such species-specific ways. Thus, mirroring Tinbergen's famous four questions (1973), Dethier pursued his research with two interacting efforts: dissecting the mechanisms by which animals accomplish their perception of the world and deriving evolutionary ideas of the behavioral and genomic consequences of that particular "world view." Such diverse views then serve as models for human biology—both by contrast and by analogy, if not homology. Building on the theme of animal world views serving as models for human perception and engineering, I will summarize some recent work on odor dynamics in which my colleagues and I have attempted to answer questions not unfamiliar to Vince Dethier (*e.g.*, 1975, 1980): How do lobsters and humans see their odor worlds?

In humans, odor evokes powerful memories—often visual images—of past experience. When humans dream of odor landscapes they may *visualize* the hills of Provence covered in fragrant thyme and lavender. On a foggy night, winds may carry the scent of pine forests and warn the sailor to *watch* or *listen* for nearby shores. And whiffs of fresh-baked bread may alert the bypasser to *look* for the bakery. For us, odor is an important and highly

specific identifier that lets us recognize which source of odor may be in proximity. In addition, odor's link with memory allows us to avoid or pursue sources of known odors. Locating a source of known odor is greatly facilitated by visual (or occasionally acoustic) exploration of the environment: the odor evokes a visual search image that we use to seek the expected odor source in a *visual* landscape. However, in the process of locating unknown odors, we are often forced to wander randomly, perhaps guided or misguided by what we consider likely locations, and then narrow our search as we encounter the odor more frequently, and perhaps more strongly. In short, when we navigate in an odor landscape we act no more efficiently than a bacterium locating patches of higher nutrient signal concentration (Adler, 1969; Berg and Purcell, 1977).

Many animals locate odor sources and for good reason. All living organisms are "leaky bags" from which odors emanate at various rates as a necessary result of metabolism; odor sources include skin, gills or lungs, urine, and feces. Wounds result in release of tissue fluids. Dead organisms become complex sources of odor produced by microbes. This rich environment is stirred by ambient currents and currents generated by organisms themselves, creating a highly dynamic spatial-temporal pattern of chemical signals and noise (Fig. 1). This spatially and temporally dynamic pattern of odor patches constitutes an odor landscape, and we wonder whether or how animals might navigate in this landscape if they were using purely chemical sensors to find important odor sources.

Natural processes are dynamic over a large range of time and space scales. Animal sensory systems are tuned to specific subranges: their filter bandwidth. If we are to understand the animal's sensory world we must first un-

Received 27 December 1995; accepted 17 April 1996.

This paper was originally presented at a symposium titled *Finding Food: Neuroethological Aspects of Foraging*. The symposium was held at the University of Massachusetts, Amherst, from 6 to 8 October 1995.



Figure 1. Normal gill current of lobster (*Homarus americanus*) visualized with dye shows typical, turbulent jet plume.

derstand its sensory bandwidths. To explore the world of underwater odor signals, we designed a number of artificial sensors that could resolve the dynamics of odor concentration with the same spatio-temporal bandwidth as the lobster, *Homarus americanus*, an animal that has guided most of our work on marine chemical signals. The essential anatomical unit for lobster chemotaxis is the aesthetasc sensillum of its lateral antennular flagellum (Devine and Atema, 1982) (Fig. 2). The final sensor we now use is a carbon-filled glass microelectrode (Gerhardt *et al.*, 1982) that matches the aesthetasc diameter of 30 μm ; we pushed the temporal resolution of the electrochemical detection process up to 200 Hz (Moore *et*

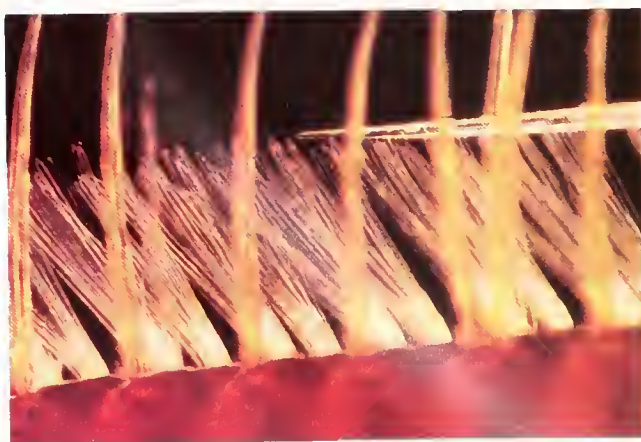


Figure 2. Glass electrode odor sensor, here placed horizontally near the tips of olfactory (aesthetasc) sensilla (rows of slanted, transparent hairs) of the lobster's lateral antennular flagellum. The electrochemical sensor, size-scaled to one lobster aesthetasc sensillum for comparable spatial resolution, detects dopamine used as a tracer for odor. Temporal resolution of sensor up to 200 Hz; of lobster olfactory receptor cells about 5 Hz.

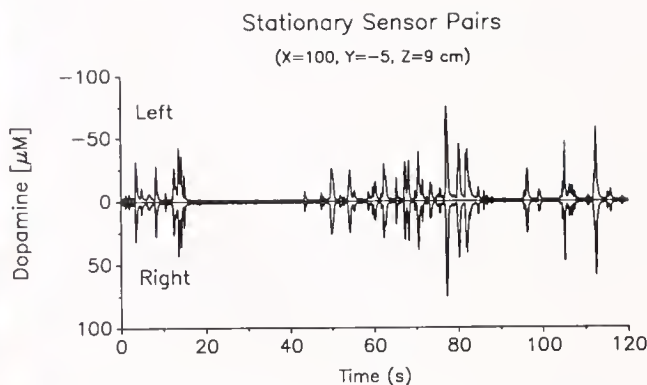


Figure 3. Typical peak structure of continuous odor (dopamine) concentration patterns measured with two stationary electrochemical sensors (30- μm tip diameter, 10-Hz sampling rate) spaced 3 cm apart at 9 cm above the ground similar to bilateral lobster antennules. The sensors are located 100 cm down-current 5 cm to the right of the center (X-axis) of the flume. Right side inverted for clarity.

et al., 1989), significantly exceeding the lobster's chemosensory flicker-fusion frequency of 5 Hz (Gomez *et al.*, 1996). Taking advantage of the sensor's original purpose (Gerhardt *et al.*, 1982), and to avoid unknown backgrounds of the natural amino acids and amines commonly detected by lobsters, we used the neurotransmitter dopamine, normally absent in seawater, as an odor tracer. This allowed us to follow the physics of odor dispersal processes without regard to the composition of the odor mixture. We measured dopamine dispersal patterns generated from a jet nozzle in a 2-m laboratory flume with a slow, parallel carrier flow of natural seawater. These dispersal conditions mimic the jet properties of the filter-feeding-breathing-excretory current of the blue mussel (*Mytilus edulis*) in a subtidal current (Moore and Atema, 1988; Moore *et al.*, 1989), as well as the lobster's own gill current (Atema, 1985) (Fig. 1). Depending on initial conditions, the turbulent dispersal process results in typical patterns of concentration peaks (Fig. 3) that can be described as statistical distributions and averages. Similar patterns are seen due to wind dispersal in air (Murlis and Jones, 1981; Murlis *et al.*, 1992). Instantaneous odor concentrations at any one place and time cannot be predicted—only a statistical range of possibilities can be determined (Moore and Atema, 1991; Dittmer *et al.*, 1995; Grasso *et al.*, unpubl.). If this is what the lobster's nose encounters, how can it extract a spatial gradient necessary for locating the odor source?

A common human solution to tackling problems involving turbulence is to take a long time or spatial average of concentration at several locations in the odor plume (Sutton, 1953). Reaching a reasonably stable average may require sampling times of several minutes at

each location (Elkinton *et al.*, 1984). A lobster would have to generate such averages at several different locations before a spatial gradient could be determined and classical (mean gradient) chemotaxis could occur. Needless to say, many odor sources (food, mates) do not last that long: the source moves, or someone else gets there first. There must be selection pressure for speed of performance. Indeed, experiments demonstrate that lobsters can find odor sources 2 m away in 30 s (Moore *et al.*, 1991). We then explored the theoretical possibility that lobsters could use a landscape (*i.e.*, typical spatial distribution) of odor eddies to make directional decisions for chemotactic orientation. I refer to this as *eddy chemotaxis* to distinguish it from classical mean gradient chemotaxis. We wanted to know the theoretical and practical limits of pure chemical orientation and navigation before exploring the very reasonable possibility that lobsters use additional sensory cues, particularly local water flow. By analogy I would call the latter process *eddy rheotaxis*: *i.e.*, steering by small-scale patterns of water flow. Although unexplored in detail, theoretical considerations of odor-dispersal processes suggest that, at least near the source of jets and wakes, flow eddies and odor patches may be coincident, providing more salient information than either odor or flow alone. This suggests that bimodal sensory cues would be used in what we could now call *eddy rheo-chemotaxis*. Bimodal sensory anatomy for chemo-mechanoreception is common in vertebrates and arthropods. It is unlikely that lobsters use sensory cues other than these two for odor-source localization.

Eddy Chemotaxis

Lobsters use bilateral antennule input for efficient direction choice and orientation; with one antennule they can still locate an odor source, but the process takes much more time and the path is convoluted. To explore the physical basis for this behavior, we measured odor-dispersal patterns in the standard jet plume with a pair of aesthetasc-sized electrodes (Fig. 2) spaced 3 cm apart (Grasso, Basil, and Atema, unpubl.), similar to the lobster's bilateral antennule spacing. These measurements have shown us three important results: (1) bilateral comparison of individual peak arrivals can show quickly (~ 1 s) which electrode is located closer to the plume's center axis—*i.e.*, the one first hit by the odor patch; (2) the rising concentration (or onset) slopes of individual peaks show a spatial gradient of increasing slope steepness toward the source; and (3) different areas in the plume show characteristic distributions of peak shapes—*i.e.*, certain shapes are more common in certain areas, creating an odor landscape that, theoretically, could be used for orienta-

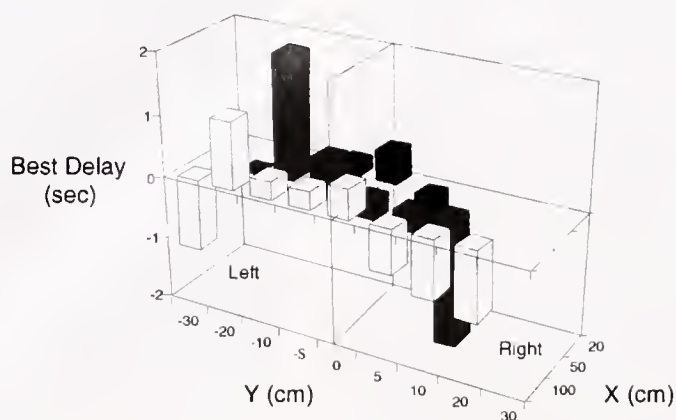
tion and navigation (Fig. 4). The results show that we can extract gradient information based on these odor-pulse shapes. Now we need to know whether lobsters detect and use that information. Before discussing the evidence and hypotheses about these three chemotaxis scenarios, we must first see if the lobsters' physical presence and their flicking and fanning behaviors disturb these patterns beyond recognition.

To test whether lobsters encounter the same patches measured in plumes without lobsters present, Basil, Grasso, and I mounted bilateral sensors in the animals' olfactory sampling area. Thus, we measured actual odor-peak patterns encountered by lobsters in the process of locating the source of an odor plume (Basil and Atema, 1994; Basil, Grasso, and Atema, unpubl.) (Fig. 5). These bilateral patterns corresponded closely to the peak distributions measured with paired sensors in similar plumes without a lobster present (Fig. 3) (Grasso, Basil, and Atema, unpubl.). Thus, the way in which a lobster walks toward the source does not significantly affect the odor patterns it faces. There may be several reasons for this. First, lobsters are surprisingly streamlined and do not create large bow waves; also their antennules are located in front and often held upward, placing them largely out of the lobster's boundary layer. Second, during the odor-tracking phase, lobsters rarely flick their antennules; flicking causes local flow fields that may or may not significantly distort measurement of existing odor-patch structure (Moore and Atema, 1988). Third, tracking lobsters use exopodite fanning to draw a gentle flow of water from the anterior hemisphere toward the head and antennules (Atema, 1985); without this exopodite fanning, the strong forward gill current (Fig. 1) would cause major flow disruption and odor-patch distortion, although these effects have not been carefully measured. Apparently, lobsters avoid distorting natural odor patterns before they reach the antennules.

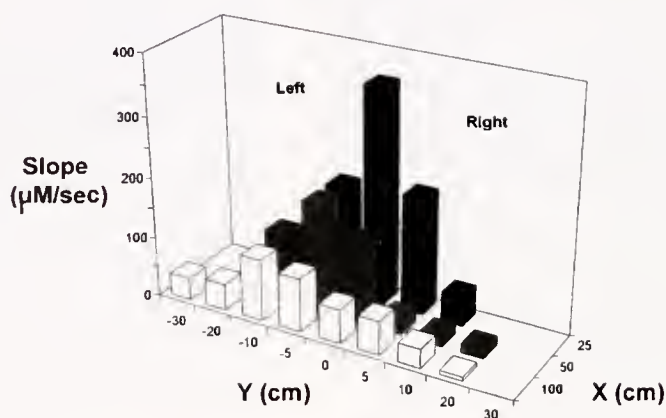
Bilateral information processing

Basil, Grasso, and I used the bilateral patterns of odor concentration patches collected from odor-tracking lobsters (Fig. 5B) for two correlation analyses. First, we established a cross-correlation between pulses measured on the left and on the right. This correlation was strongest with a time shift of 0.5–1 s (Grasso, Basil, and Atema, unpubl.). We interpret this to reflect the passing of single odor patches across one and then the other antennule with a delay of 0.5–1 s. Lobsters could use this information to steer toward the plume's main axis because odor patches tend to move laterally out from the main axis. Thus a left hit followed in about 0.5–1 s by a (similarly shaped) right hit should result in left-turn

A - Best Left-Right CCF Delay



B - Average Peak Slope



C - Odor Landscape

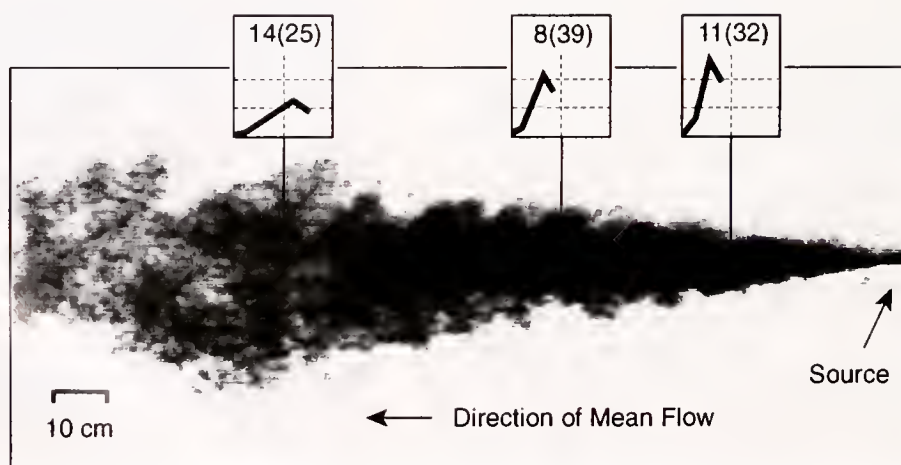


Figure 4. Sources of directional information in a chemical jet plume that could be used in eddy chemotaxis. (A) Spatial distribution of "best delays" in cross-correlation functions (CCF) between left and right stationary electrodes spaced 3 cm (*cf.* lobster antennule spacing) perpendicular to mean flow measured at 21 flume positions. On the right side of the flume X -axis ($Y = 0$), left hits precede right hits by 0.3–2 s; the reverse occurs on the left side. This information may allow the lobster to make quick turning

behavior and *vice versa*. Simultaneous hits should result in no turn. A second cross-correlational analysis supports this hypothesis (Basil, Grasso, and Atema, unpubl.). We calculated the instantaneous left-right concentration differences measured between bilateral sensors on the lobster every 1 s and established a cross-correlation between this difference signal (the animal's chemosensory input) and lobster turning angles (the animal's behavioral output) (Fig. 5c). The best cross-correlation of about 0.6 occurred when the turning behavior lagged the difference signal by about 2–4 s. In other words, lobsters responded with an appropriate turn after a delay of a few seconds. Although correlational, these results support the notion that bilateral odor-pulse input is steering lobster behavior continuously (Basil, Grasso, and Atema, unpubl.).

The difference signal includes time-correlated bilateral hits from the same passing odor patch and uncorrelated unilateral hits on either side. The correlated hits may allow for quick turning responses as argued above, whereas more time may be required to evaluate the uncorrelated accumulation of hits on one side exceeding those on the other side. The latter may result in the 2-s turning delays. It may also explain why tracking lobsters walk at half or less their maximum speed: any faster and they might lose the track (Moore *et al.*, 1991; Basil, Grasso, and Atema, unpubl.). These results are consistent with results from unilateral lesion studies which show that animals with one antennule cannot make correct turning decisions; eventually they do reach the source, but follow a tortuous path (Devine and Atema, 1982). This path must result from sequential analysis by a single antennule (with a little help from bilateral dactyl receptors on walking legs; Devine and Atema, 1982).

Spatial gradient of peak onset slopes

Analysis of individual peak features has shown consistently that the best spatial gradients are extracted from the spatial distribution of the onset slopes (Fig. 4B) and heights of the peaks, whereas peak duration and intermittency are unreliable gradient features on the time scale of the lobster (Moore and Atema, 1991; Grasso, Basil, and Atema, unpubl.). These physics results have in-

teresting physiological correlates. Neurophysiological analysis of olfactory chemoreceptor cell responses to carefully controlled odor pulses has shown that these cells integrate over 0.2 s, fully adapt in about 1 s, recover partially in 1–5 s and fully in 15–25 s, and have flicker fusion frequencies of 1–5 Hz (Gomez *et al.*, 1994; 1996; Gomez and Atema, 1996a,b). In addition, background stimuli shift the response function to higher concentrations (Borroni and Atema, 1988), and repeated stimuli result in cumulative adaptation (Voigt and Atema, 1990). Thus, antennular chemoreceptor cell responses show bandwidth properties favoring a temporal spectrum of fast events that match common odor-pulse onset times and rejecting slower concentration changes. These properties would favor detection of pulse arrivals in the subsecond range above ambient backgrounds that may be fluctuating in the 10-s range and longer.

It is tempting to infer that these physiological filter properties evolved to exploit the most useful information contained in turbulent odor plumes, driven by the competitive advantage of locating sources of odor before someone else does. To use the spatial distribution of peaks as an aid in orientation, an animal would have to recognize different peak onset slopes because it is the spatial gradient of onset-slope values that contains the best directional information. The spectrum of different temporal tuning properties of lobster antennular chemoreceptor cells (Gomez *et al.*, 1996; Voigt and Atema, 1989) support this possibility: as different cells are differentially excited by different slopes, a population (*i.e.*, “across-neuron”) code of slope information could be used to measure slope values. As lobsters move through a plume, they could monitor the sequence of slope values and steer a direction in which slope values increase most. This would be the “best route” to the source (Fig. 4B).

Thus, we hypothesize that lobsters sense whether onset-slope values of odor patches increase or decrease as they are being encountered; this would allow the lobster to make estimates of best turning directions while walking. Arriving at acceptable estimates may take some time, and this behavioral integration time may regulate the (low) maximum walking speeds of lobsters during chemotaxis (Moore *et al.*, 1991; Basil, Grasso, and

decisions toward the *X*-axis. (B) Spatial distribution of average odor-peak onset slopes measured at the same 21 flume positions as in A: slope values increase towards the source of odor release, both in *X* and *Y* directions. This information could be used for sequential comparison to determine direction to the source. (C) “Landscape” of odor-peak shapes: visual projection of dye plume is shown with predominant locations of selected common peak shapes (insets). Peak shapes are constructed from mean values of parameters describing peak classes determined by Bayesian classification of measured peak parameter values. Inset calibration lines: 1 s horizontal, 100 μ M vertical. Numbers in insets indicate class label and, in parentheses, number of peaks in each such class.

Atema, unpubl.): walking speed may be optimized for peak encounter rates. If so, walking speeds should vary with eddy-dispersal conditions in different kinds of plumes.

Such inferences are now being tested directly with both real and robotic lobsters. Real lobsters are equipped with head gear delivering known odor pulses to bilateral antennules, and the resultant turning behavior is monitored. Thus, by creating a virtual reality of controlled bilateral odor pulses, one should be able to "lead the lobster by the nose" toward an imaginary odor source. This requires delivering bilateral series of specific odor pulses, preferably with controlled shapes, in the olfactory sampling area of freely walking lobsters. In addition to these lobster tests, we are testing the limits of eddy chemotaxis with the aid of a robot model, "RoboLobster," which is built on some of the known principles of chemoreceptor filter properties, sensor size and spacing, and locomotion and turning speed in lobster (Consi *et al.*, 1994, 1995) (Fig. 6). Because we can choose to provide the robot only with bilateral chemosensory information, we can exclude rheotaxis. The robot allows us to explore the spatial resolution and potential of pure chemotaxis as well as rheotaxis under a variety of plume conditions and sensory filter algorithms.

Odor landscapes

As indicated by a Bayesian classification* of the odor-pulse shapes we measured during turbulent dispersal, certain plume areas are reliably characterized by particular pulse shapes (Grasso, Basil, and Atema, unpubl.) (Fig. 4c). This represents an odor landscape and thus contains the possibility of navigating through it. Theoretically, one could enter this landscape at any point and—when it is a familiar landscape—know how to get to its highest peak: the odor source. Navigating through a landscape is different from sequential and bilateral steering along a gradient of peak onset slopes, primarily in that it presupposes prior knowledge of the odor landscape. Most animals reside in specific ocean realms and may well be familiar with various flow regimes and dispersal patterns. Thus, theoretically, they could navigate through an odor-peak landscape. This landscape is defined exclusively by the local odor-concentration fluctuations that vary from place to place, with peaks getting

*The Bayesian process is a fuzzy classifier that organizes the seemingly random assortment of peak shapes into more-or-less distinct classes; it maximizes the likelihood that the classification accounts for the data and that the data fit the classification. Thus both the number of classes and the probability of membership in a particular class are optimized (Berger, 1985).

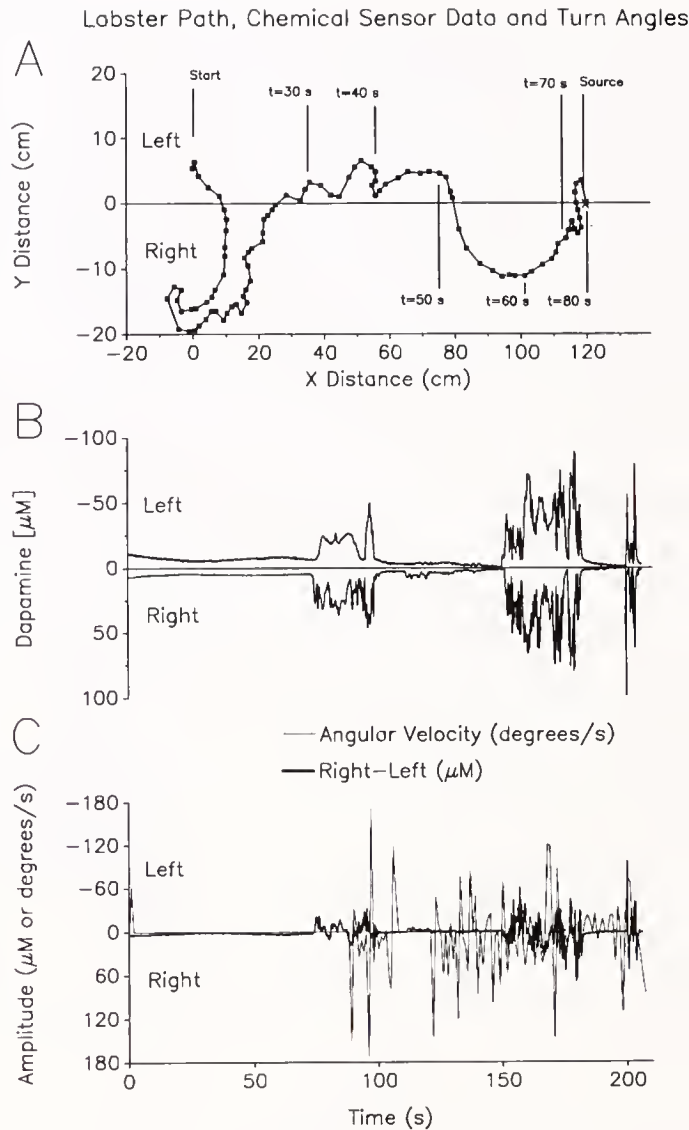


Figure 5. (A) Lobster track during odor-plume source localization, digitized in 1-s steps. Time marks correspond to sensor data in B and C. (B) Bilateral measurements of odor-tracer (dopamine) concentration from electrochemical sensors in antennule sampling area made during successful source localization in A. (C) Bilateral (right-left) difference signal from dopamine sensors (5B) superimposed on angular velocity of lobster turns from 5A: input leads output by a few seconds; many inputs do not result in turns, and vice versa.

steeper and taller near the source. We might add that additional features of odor landscapes may include changes in odor composition. After release, mixture components may be removed differentially—and perhaps predictably—due to bacterial uptake, adsorption, photolysis, etc. If predictable, these qualitative changes could aid in navigation. Finally, over larger distances certain areas, such as river mouths and weed patches (as well as laven-

der bushes in Provence), may be reliably identified by their odor quality, thus providing aid to navigation by local flavor. In this discussion, which is focused on spatio-temporal fluctuations in odor concentration, we do not further consider the two landscape forms based on odor quality.

Eddy Rheotaxis

Classically, rheotaxis refers to directed locomotion guided by the mean flow vector; most commonly and roughly this is up-current movement. However, because many objects in moving fluids (and moving objects in fluids) leave behind a vortex trail, these turbulent wakes may provide a hunter with directional information about source location based on sequential analysis of eddies, if these eddies are about the size of the hunter or smaller. We visualized such a wake for a salp and hypothesized that commensal shrimp may use this wake to locate their host (Diebel *et al.*, 1985). This form of—thus far hypothetical—rheotaxis may be referred to as *eddy rheotaxis*. Given the ubiquity of wind and currents it would probably not pay to respond to just any flow, and wakes may have to be structured quite specifically to allow hydrodynamic receptors to identify the source so that the hunter can decide whether to pursue and locate the signal or to ignore it. However, as soon as wakes and eddies are “flavored” with the exudates of the source, organisms can establish source identity by chemical signature and then use rheotaxis to locate the source. This would be odor-triggered rheotaxis (*e.g.*, Zimmer-Faust *et al.*, 1995).

Eddy Rheo-Chemotaxis

Even more informative may be the use of flavored eddies in a wake. These could be analyzed simultaneously by chemo- and mechanoreceptors, leading to *eddy rheo-chemotaxis*: steering toward a source of odor and turbulence using flavored eddies detected simultaneously by chemo- and mechanoreceptors. The receptors in question are primarily olfactory chemoreceptors and hydrodynamic flow receptors such as the lateral line of fishes and equivalent structures in arthropods and other invertebrates. Many arthropod sensilla are bimodally innervated by chemo- and mechanoreceptor cells (Altner and Prillinger, 1980) that can respond at the same time and place to the arrival of a flavored eddy. Inertial detectors such as fish otolith organs and invertebrate statolith organs may also be involved when eddies are about the size of the animal or larger. In special cases, such as the catfishes with tastebuds covering their body surface, taste receptors are involved in chemotaxis (Bardach *et al.*, 1967; Atema, 1971). What is of particular interest here is

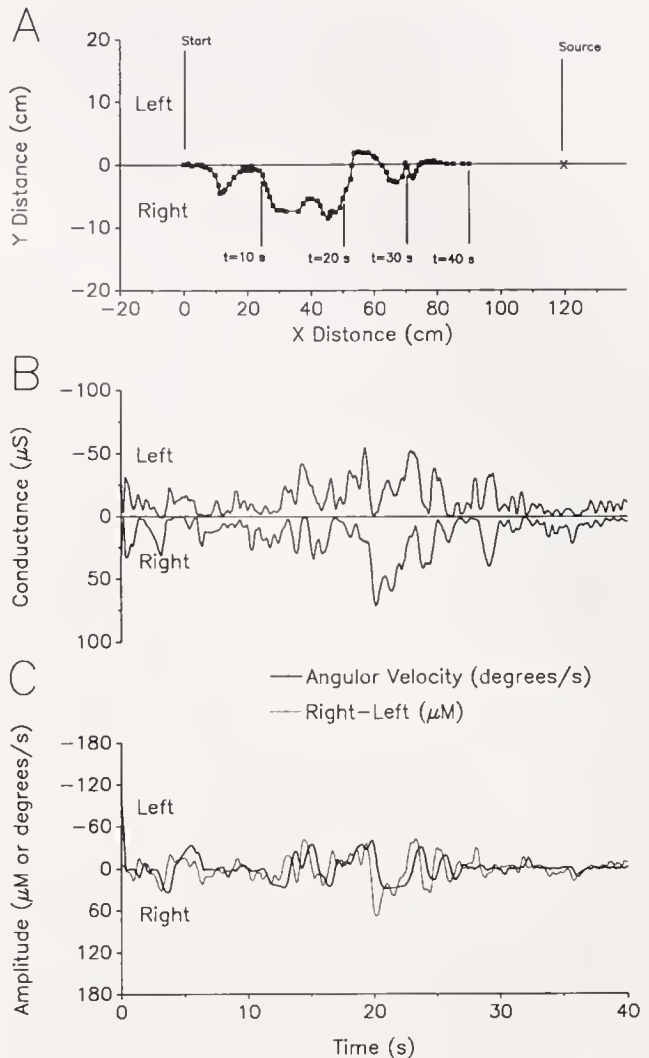


Figure 6. (A) Robot track during salt-plume source localization digitized in 0.3-s steps, as executed based merely on the signal provided by the instantaneous input difference in bilateral salt concentration (6B). Time marks correspond to sensor data in B and C. (B) Bilateral measurements of salt concentration from conductivity sensors on robot during successful source localization in A. Right side inverted for clarity. (C) Bilateral (right-left) difference signal from conductivity sensors (6B) superimposed on angular velocity of robot turns from 6A. Difference signal is used to steer robot output in salt plume: input systematically leads output by about 0.5 s. Note: salt-plume dispersal in fresh water is comparable to the dopamine-plume dispersal in seawater used in lobster behavior and other experiments.

the spatial and temporal resolution of both sensory modalities and the coincidence with which the chemical and mechanical information is extracted. Flavored eddies provide such coincident information.

Given the ubiquity of body odors, wind, and currents, it is probable that eddy rheo-chemotaxis will be used by many animals. It should be particularly important in

mid-water, deep-water, and open-ocean environments and in dark terrestrial environments such as caves or rain forests at night. In these habitats, it is difficult to establish an external reference for measuring drift with the prevailing currents. Flying moths look at the ground to measure drift, allowing them to respond to the local wind direction at every fraction of a second (David *et al.*, 1982). Open-ocean animals and animals in visually limited environments may not be able to measure this drift because their entire measurable world is drifting with them. Therefore they could not visually navigate "up-current" as known in moths (Mafra-Neto and Cardé, 1994; Vickers and Baker, 1994) and shallow-water fish (Hodgson and Mathewson, 1971; Kleerekoper *et al.*, 1975). Eddy rheo-chemotaxis is independent of this large-scale drift because the drifting plume itself contains all relevant information.

Many events in an animal's local environment create eddies, and many of these eddies may be variously flavored depending on the organisms that generated them. Thus, the signal eddies can be screened from the noise eddies on the basis of the specificity afforded by body odors (any odor leaking from an organism), including pheromones (specialized communication signals). Under special circumstances, eddy flow alone may generate sufficient source specificity that added flavor is not needed to identify and locate the source.

Sources of odor dispersal vary. Many animals generate their own feeding, breathing, and excretory currents, which generally take the form of *jets* (e.g., Fig. 1). In a jet, the initial energy of dispersal is dominated by the jet; further away from the source, as jet energy becomes dissipated by viscosity, ambient currents begin to match jet flow velocities and thus more and more begin to influence dispersal, resulting in a loose patch field of flavored eddies. The filter currents of bivalve molluscs and the gill currents of decapod crustaceans are good examples of jets. Lobsters can inject urine into the gill current (Atema, 1986); the urine in turn may be spiked with the product of a specialized gland (Bushman and Atema, 1993, 1996). Although both the prey odor (Derby and Atema, 1981) and the urine pheromone currents (Atema, 1995) contain important chemical signals for the lobster, we do not know if the fine structure of these jet plumes contributes information critical for determining the identity and direction of the source. It is likely that jet-dominated dispersal areas provide better directional information than the more distal "patch field" of odor and eddies. In the jet area, dispersal patterns of odor patches and eddy flows may show a high degree of correlation, because the highest energy eddies are flavored from birth at the jet nozzle. Moreover, eddy flow directions are more predictable in jets, as exemplified beautifully in smoke rings and in the series of bilat-

erally alternating eddies known as vortex streets, that spin off behind a moving object. Somewhat similar dispersal patterns are generated by *wakes*. Wakes result from "active" locomotion and from "passive" ambient currents washing over an odorous body: the wake behind the body becomes flavored and the dispersal pattern is determined by the shape of the body and the flow around it. Wakes and jets, beyond the distance in which jet energy dominates, will disperse similarly by ambient currents.

Eventually, odor-patch gradient information may become important independently of flow, as eddy flow may dissipate before odor patches become fully mixed. In that case, the longevity of the odor patch becomes the dominant factor: the longer a drifting odor patch remains identifiable against the background, the more its edges will have sheared and diffused, so that its edge concentration slopes become ever shallower farther away from the source. If animals can detect these concentration-patch slopes, they could sample a series of patches to determine in which direction their patch slopes increase most: this direction would lead them toward the source. Thus, without any further sensory information, an animal might sample a few random tracks through an odor-patch field and determine the direction in which patch slopes increase most dramatically. This could be done at different time and space scales by animals of different sizes and locomotor capabilities.

Finally, some functional constraints argue indirectly for a more general use of eddy rheo-chemotaxis. When external references, such as visual or (electro)magnetic measurement (Kalmijn, 1988) of drift, are not available, navigating may become limited to following a statistically reliable sequence of local sensory cues that generally lead the animal to the source of odor release. A wake of eddies, particularly flavored eddies, may be all that is left for orientation. Thus, in one form or another, an eddy rheo-chemotaxis mechanism may not be limited to dark and murky environments under water or in air. Even in clear oceanic water, visual range is limited to a few hundred meters due to scatter. For large predatory fish this is not very far. Because eddies and turbulent wakes can exist over a wide range of space and time scales—ultimately up to the size of ocean basins—large and fast animals such as tuna could use eddy chemotaxis or chemo-rheotaxis to bring them within range of other sensory cues. They could sample odor patches at a 1-s time scale and a 10-m size scale in pursuit of large prey schools several kilometers away. Even in the open ocean, prey can hide visually to some extent, but a temporary chemical signature is left inevitably in the wake of anyone's presence. If the eddy flow of a wake dies out before the chemical signature melts away, it would leave pure eddy chemotaxis as the last remaining mechanism to lo-

cate biologically important targets. Pigeons (Papi, 1976) and oceanic birds (Nevitt *et al.*, 1995) may use similar mechanisms. (I have deliberately omitted a discussion of long-range acoustic prey detection and sonar used by marine mammals, aerial bats, and other acoustically specialized animals.)

Conclusion

Despite the physical evidence and physiological filter correlates presented here, we are a long way from demonstrating that animals use eddy (rheo)chemotaxis. The physics of dispersal measured with animal-sensor-scaled odor sensors, and the dynamic filter properties of lobster chemoreceptor cells and organs make it plausible that eddy chemotaxis can be an important guidance mechanism for odor-source localization. Moreover, behavioral experiments, including track analysis and cross-correlations between bilateral chemical signal detection and turning angles, so far do not refute the hypothesis that flavored eddies are used in this localization process, at least over short but behaviorally critical distances. 'RoboLobster' is beginning to provide independent data on pure eddy chemotaxis. Eddy rheotaxis may turn out to be an important contributor to plume navigation. Direct demonstration that odor patches and flow eddies steer animals toward the odor source is still absent. Whereas it seems likely that various animals use eddy (rheo)chemotaxis, it seems unlikely that humans have this capability. This leaves us to enjoy odor quality landscapes and to admire various animals for their superhuman perceptions of Nature.

Acknowledgments

I thank my colleagues and collaborators cited throughout this paper for their valuable discussions and critique, in particular Drs. Jennifer Bail, Thomas Consi, George Gomez, Frank Grasso, and Rainer Voigt. Postdoctoral Fellows Jennifer Basil and Frank Grasso, in particular, contributed significantly to the bilateral information data and ideas expressed in the text and Figures 3–6. Photo credit Fig. 1: Linda Golder, MBL. The research which forms the basis of this paper was financially supported primarily by NSF grants IBN-9222774, IBN-9212650, OCE-9315083, and BES-9315791.

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Flies, Genes, and Memory Engineering

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Introduction

Perhaps these insects are little machines in a deep sleep, but looking at their rigidly armored bodies, their staring eyes, and their mute performances, one cannot help at times wondering if there is anyone inside.

—Dethier (1964)

With these words Vincent Dethier closed his essay on “Microscopic Brains” in which he argued that the behavior of insects and vertebrates may not differ qualitatively to the extent that had been supposed (Dethier, 1964). Subsequent work on learning in insects and on the biochemical algorithms that implement learning has demonstrated the prescience of his vision.

The foraging and feeding behaviors of flies have proven particularly useful for studies of learning relevant to several different problem areas, *e.g.*, optimal foraging theory (Mangel and Roitberg, 1989; Mangel, 1993), reproductive fitness (Tompkins *et al.*, 1983; Greenacre *et al.*, 1993; Griffith *et al.*, 1993; Hall, 1994; Prokopy *et al.*, 1994), visual processing (Dill *et al.*, 1995; Fukushi, 1994), and biochemical mechanisms of memory storage (Feany and Quinn, 1995; Greenspan, 1995; Yin and Tully, 1996). Recent work with genetically engineered flies shows that long-term memory formation can be greatly augmented if selected single genes are activated prior to training (Yin *et al.*, 1995). This result encourages the speculation that gene therapy, applied in humans to regions of the brain that are crucial for memory function (Naldini *et al.*, 1996), may provide an alternative to current pharmacological (Staubli *et al.*, 1994) or surgical (Martinez-Serrano *et al.*, 1995) interventions for repair or enhancement of memory function (Carew, 1996).

Flies Can Learn

Flies are capable of both associative and nonassociative learning, making use of a variety of sensory input

channels (olfactory, visual, mechanosensory) that are coupled to behavioral readouts such as flying, walking, courtship, and the proboscis extension reflex (Spatz *et al.*, 1974; McGuire, 1984; Dudai, 1988; Prokopy and Papaj, 1988; Heisenberg, 1989; Troje, 1993; Fukushi, 1994; Hirsch and Tompkins, 1994; Kyriacou and Hall, 1994). The learning ability of the black blowfly, *Phormia regina*, is a subject of particular interest (Akahane and Amakawa, 1983) because more is known about the regulatory mechanisms governing feeding behavior in this species than any other invertebrate (Dethier, 1976; Gelperin, 1987). Classical conditioning in *Phormia* was demonstrated after repeated pairings of an unconditioned stimulus (sugar) with a conditioned stimulus (water or saline) (Nelson, 1971). The statistical nature of the learning left open the question as to whether learning plays a role in the normal foraging behavior of *Phormia*. In a subsequent study, in which the odor-shock training procedure used in studies of *Drosophila* learning was applied to *Phormia*; significant learning was produced but at a level lower than that found in the Canton-S strain of *Drosophila* (McGuire *et al.*, 1990).

Blowflies show robust and reliable learning, however, when trained during the “dance” that follows the ingestion of a subsatiating drop of sugar water (Dethier, 1957; see also Bell, 1985). When a hungry fly ingests a small drop of concentrated sugar solution presented on a colored background, its subsequent searching behavior is guided by color-food associative learning (Fukushi, 1985, 1989, 1990, 1994). Because blowflies visit nectar-bearing flowers (Kearns and Inouye, 1993, 1994), and the color-food association is strong after a single training trial, color-nectar learning plausibly plays a role in the foraging behavior of wild *Phormia*.

The analytical power of neurogenetics and its application to diverse vertebrate and invertebrate species allowed studies of fly learning to make major contributions to understanding memory mechanisms. While work with *Phormia* focused on how learning modified

particular aspects of foraging or feeding behavior of individual flies, neurogenetic studies of *Drosophila* learning used a behavioral assay for learning developed as a selective agent applied to groups of flies after chemical mutagenesis. The development of new tools for molecular genetic analysis and their application to studies of learning in *Drosophila* and mammalian species have revealed a remarkable congruence between invertebrates and mammals in both the logic operations implemented behaviorally and the biochemical mechanisms used for coincidence detection at synapses (Carew and Sahley, 1986; Gelperin *et al.*, 1986; Byrne, 1987; Gould, 1990; Krasne and Glanzman, 1995; Sahley, 1995; Menzel and Müller, 1996). Recent studies in *Drosophila*, *Aplysia*, and *Mus* have been particularly suggestive of a common mechanism for long-term memory among arthropods, molluscs, and mammals (Kandel and Abel, 1995; Yin and Tully, 1996).

Neurogenetic Analysis of Learning

Neurogenetic analysis of learning in *Drosophila* became possible when a reliable method for training large numbers of flies to associate odor with foot shock was developed (Quinn *et al.*, 1974; Tully and Quinn, 1985). During training, groups of flies were exposed to an odor-impregnated circuit board with parallel conductive strips that allowed a foot shock to be applied during exposure to an odor (odor A). A second odor (odor B) was presented without shock application. During testing, the group of trained flies was conveyed to a choice point where they could enter a tube containing odor A or a tube containing odor B. A learning index was computed as the fraction of flies choosing odor B minus the fraction choosing odor A; the learning index was averaged over this experiment and a reciprocal experiment in which odor B, not odor A, was paired with shock. The average learning index for discriminative classical conditioning of the Canton-S strain is 0.9, indicating that 95% of the flies avoided the shock-associated odor (Tully and Quinn, 1985).

Use of the odor-aversion learning paradigm as a screen after chemical mutagenesis led to isolation of the first learning mutant, *dunce* (Dudai *et al.*, 1976). Several other learning mutants, *turnip*, *rutabaga*, *cabbage*, *radish*, and *amnesiac*, were identified subsequently (Quinn *et al.*, 1979; Livingston *et al.*, 1984; Dudai, 1988; Folkers *et al.*, 1993; Tully *et al.*, 1994). Memory formation in *Drosophila* displays a seemingly universal characteristic; it has a short-term, easily disruptible form that is converted within a few hours to a long-lasting, more stable form (Quinn and Dudai, 1976; Menzel *et al.*, 1990; Zola-Morgan and Squire, 1993). Long-lasting memory can be further subdivided into different phases based on differential sensitivity to cooling after memory reactivation (Yamada *et al.*, 1992) or pharmacological treatments

that distinguish protein synthesis-dependent and independent memory (Yin *et al.*, 1994). By modifying the original aversion learning assay, both appetitive learning by learning mutants (Tempel *et al.*, 1983) and learning by larvae as well as adults (Aceves-Piña and Quinn, 1979; Tully *et al.*, 1994) could be assessed. Mutant phenotypes have selective deficits in acquisition, short-term retention, or long-lasting memory retention (Quinn and Greenspan, 1984; Tully *et al.*, 1990; Davis, 1993; Tully and Gold, 1993; Greenspan, 1995). New techniques for mutant production, such as P element mutagenesis, have added to the stock of memory mutants and have greatly facilitated the identification of mutant gene products (Boyton and Tully, 1992; Dura *et al.*, 1993, 1995; Bolwig *et al.*, 1995).

Cyclic AMP and Memory Formation

Three of the genes essential for normal memory function in *Drosophila* code for products affecting intracellular levels of adenosine 3', 5'-monophosphate (cAMP). The *dunce* gene encodes a cAMP-specific phosphodiesterase (Qiu *et al.*, 1991). The *rutabaga* gene encodes a calcium/calmodulin-sensitive adenylate cyclase (Dudai and Zvi, 1984; Levin *et al.*, 1992; Livingston *et al.*, 1984). Finally, the defective gene in the memory mutant *amnesiac*, which learns normally but forgets rapidly, encodes two neuropeptides; one of these peptides has substantial homology to mammalian pituitary adenylyl cyclase activating peptide (PACAP) (Feany and Quinn, 1995; Zhong and Pena, 1995). The involvement of these three genes in the control of cAMP levels has fueled speculation that a common mechanism subserves synaptic coincidence detection in flies, snails, and mammals (Kandel and Abel, 1995).

A role for cAMP in memory storage is particularly clear in *Aplysia* (Kandel and Schwartz, 1982; Kaang *et al.*, 1993; Alberini *et al.*, 1995) and *Drosophila* (Davis *et al.*, 1995; Spatz, 1995). The regulation of cAMP levels plays a role in the learning that occurs in *Aplysia* when synaptic efficacy between the sensory and motor neurons of the gill- and tail-withdrawal reflexes is facilitated (Kandel and Schwartz, 1982; Byrne *et al.*, 1993; Byrne and Kandel, 1996). Both serotonin and peptides have modulatory effects on synaptic efficacy in *Aplysia* acting via cAMP (Hawkins *et al.*, 1993; Jarrard *et al.*, 1993). A nuclear transcription factor—the cAMP response element binding protein (CREB)—serves as a link between short-term elevations in cAMP and long-term memory, which involves transcriptionally dependent processes, *e.g.*, growth of new synapses (Bailey and Kandel, 1994). In *Aplysia*, oligonucleotides containing cAMP-responsive element sites were injected into the nucleus of a sensory neuron, and this treatment specifically blocked long-term facilitation of the synaptic connection be-

tween that sensory neuron and a motoneuron in culture (Dash *et al.*, 1990). Also, stimuli that cause long-term facilitation activate cAMP responsive genes (Kaang *et al.*, 1993). When an antibody to *Aplysia* CREB is injected into a sensory neuron, a single pulse of serotonin can then evoke long-term facilitation, rather than the short-term facilitation seen in untreated and control sensory neurons (Bartsch *et al.*, 1995). In *Drosophila*, a CREB gene isoform which represses cAMP-stimulated transcription was placed under the control of a heat-shock promotor so that its repressive effects could be induced at various times after odor conditioning (Drain *et al.*, 1991). Induction of repressor isoform expression after olfactory learning specifically and completely blocked a cycloheximide-sensitive component of long-lasting memory but had no effect on original learning or on a second, cycloheximide-insensitive, component of long-lasting memory (Yin *et al.*, 1994). In separate experiments with the same genetic strategy but with the induction of an activator CREB isoform, long-term memory formation was enhanced (Yin *et al.*, 1995).

Synaptic coincidence detection may arise from the calcium/calmodulin sensitivity of the adenylyl cyclase (Yovell *et al.*, 1992), which can be stimulated both by calcium influx during action potentials and by G_s , the stimulatory guanine nucleotide-binding protein that couples stimulatory receptors to the catalytic subunit of adenylyl cyclase. If *Drosophila* neurons expressing the PACAP peptide are activated by the shock used as an unconditioned stimulus during training and if PACAP receptors activate G_s , the analogy between synaptic mechanisms for learning in the fly and snail would be compelling. Similar mechanisms are expected to play a role in the mammalian brain (Bourne and Nicoll, 1993; Cooper *et al.*, 1995; Kandel and Abel, 1995), where regions implicated in memory storage express the calmodulin-sensitive adenylyl cyclase at relatively high levels (Xia *et al.*, 1991). A transcriptionally dependent late phase of long-term potentiation in hippocampus is induced by cAMP analogs (Frey *et al.*, 1993). However, mice with a null mutation in the gene encoding a regulatory subunit of cAMP-dependent protein kinase showed a severe deficit in long-term depression, but normal late-phase LTP (Brandon *et al.*, 1995). This suggests that multiple mechanisms for cAMP dependency are subserving different forms of synaptic plasticity.

Locus of Fly Memory

The mushroom bodies of the *Drosophila* brain (Dujarin, 1850; Power, 1943) seem to be important sites for multisensory integration and memory storage; these conclusions are based on structural studies (Heisenberg, 1980; Technau, 1984; Heisenberg *et al.*, 1985) and effects on memory storage produced by localized cooling of these

structures in honeybee (Erber and Menzel, 1980). More recently, several of the genes first identified in learning mutants as regulating cAMP levels were found to have enhanced expression in the mushroom bodies (Nighorn *et al.*, 1991; Davis, 1993). Chemical ablation of the mushroom bodies abolished associative odor learning (de Belle and Heisenberg, 1994). Examination of enhancer-trap expression patterns within Kenyon cell processes in the neuropil of mushroom bodies has revealed an entirely new level of anatomical complexity (Yang *et al.*, 1995). Unraveling the computational significance of this anatomical complexity will be aided by study of mushroom body neurons and networks in culture (Wright and Zhong, 1995) and by worldwide contributions to Flybrain, an on-line atlas and database of the *Drosophila* nervous system (Armstrong *et al.*, 1995).

More than thirty years after the publication of "Microscopic Brains," analyses of targeted single gene mutations in fly and mouse suggest that the biochemistry of arthropod algorithms may indeed be used to implement some types of mammalian memory storage (Kandel and Abel, 1995; Yin and Tully, 1996).

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**Marine
Biological
Laboratory
Woods Hole
Massachusetts**

**Ninety-Eighth Report
for the Year 1995
One-Hundred and Seventh Year**

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Report of the Director and Chief Executive Officer

The Marine Biological Laboratory and its research and training programs enjoyed another successful year in 1995. Scientists working in the MBL's year-round research program continued to make important contributions to our understanding of basic biological, biomedical, and environmental sciences. Investigators coming to the MBL to work for short periods throughout the year, and especially during the summer months, provided their own invaluable insights into the workings of fundamental life processes. And the scores of students who worked day and night in the teaching laboratories of the Loeb and Lillie buildings added yet another level of excitement and curiosity to the Laboratory.

All of these scientists, whether year-round or summer, tenured professor or fledgling graduate student, came together in Woods Hole to experience the unique atmosphere of the Marine Biological Laboratory. Here they shared, studied, and grew together as scientists, all with a single goal in mind: to learn a little bit more about the world around them, using as models a variety of aquatic organisms found in the Woods Hole area.

Research at the MBL

The Marine Resources Center

Since its construction in 1992, the MBL's Marine Resources Center has developed into an important facility for maintaining and culturing organisms and for conducting biomedical research. Throughout the year, the facility now enjoys a high level of activity.

Last September, Dr. Roger Hanlon joined the MBL scientific staff as the Director of the Marine Resources Center. Hanlon comes to the MBL from the University of Texas Medical Branch (UTMB) in Galveston where he served as Chief, Division of Biology and Marine Resources at UTMB's Marine Biomedical Institute and Professor in UTMB's School of Medicine. Hanlon's neurobiological and behavioral research on

cephalopods—marine animals that include cuttlefish, squid, and octopus—adds another exciting component to the MBL's research programs. One series of his experiments is designed to determine whether cephalopods are capable of learning by observing the behavior of others, a feat requiring cognitive abilities thought to be performed only by evolutionarily advanced organisms such as birds or mammals. Hanlon's investigations on sexual selection, sperm competition, and DNA fingerprinting in squid will improve our understanding of animal behavior and will assist in efforts at improved fisheries management for a better, more sustainable catch, currently valued at \$33 million/year.

In 1995, Hanlon launched several biomedical research initiatives that employ the latest techniques in molecular biology using the cuttlefish, the Hawaiian squid, and the zebrafish as models. The newest model at the MBL, the Hawaiian squid, *Euprymna scolopes*, hosts a colony of *Vibrio* bacteria in its light organ. Understanding how the symbiosis between *Euprymna* and *Vibrio* works will provide scientists, including immunologists, with information about how all bacteria and other microbes—whether harmless or pathogenic—are able to recognize and colonize specific tissues in its host.

Program in Comparative Molecular Biology and Evolution

In 1995, the MBL's Molecular Evolution Program was renamed the Program in Comparative Molecular Biology and Evolution, a more accurate description of the program's research. Under the direction of Dr. Mitchell L. Sogin, program scientists use the tools of comparative molecular biology to study the evolution of microbes and other organisms that typically do not have a fossil record or distinctive morphology. Sogin and his colleagues compare the genes of these organisms and create phylogenies (genealogies on an evolutionary

time scale) as a means of learning more about the origin of the earliest eukaryotes (organisms, including plants, fungi, and animals, whose cells contain nuclei). By understanding the genetic makeup and relatedness of these organisms, scientists are better able to design diagnostic probes that detect disease-causing pathogens and to identify the appropriate model system to study disease processes in a variety of more complex organisms, including humans.

Last year, Sogin and his colleagues used an imaginative and productive logic to study the 'minimal' eukaryotic cell using Microsporidia as their model. Microsporidia are pathogens that infect a variety of hosts, including human and fish nerve cells; they also have the smallest genomes of any known eukaryote. By analyzing microsporidial genomes, Sogin and his colleagues hope to develop a fundamental genetic blueprint for all eukaryotes which, when compared with DNA sequences from other organisms, will reveal clues about the function and structure of a variety of genes.

The Ecosystems Center

The Ecosystems Center, co-directed by Drs. Jerry Melillo and John Hobbie, celebrated its 20th anniversary in 1995. The anniversary was an opportunity for Center staff to reflect on the remarkable accomplishments of the past and to begin assessing the Center's role for the future. In 20 years, the Ecosystems Center has grown considerably, both in terms of staff and breadth of research.

Ecosystems Center scientists study the structure and function of ecological systems. Their research takes them to remote field sites in Alaska, Brazil, and Sweden, as well as to more local study sites in southeastern Massachusetts. Although the ecosystems may vary in

size and location, the research questions addressed by Center scientists are often quite similar, regardless of the site. In 1995, the Center's projects continued to focus on studying the effects of change on ecosystems and on developing basic principles about how ecosystems work. How does the deposition of acidic compounds from factory and automobile emissions affect forests, lakes, and streams in North America? How do changes in land use affect the flow of nutrients and organic matter into New England estuaries and thus the food chain? How will the clearing of tropical rainforests change the amount of carbon dioxide released into the atmosphere? How would warmer temperatures—or a change in the global climate—affect arctic ecosystems?

Ecosystems Center scientists work collaboratively on these projects, bringing together a wealth of expertise in a variety of areas. As a result, computer modelers work with geochemists who work with terrestrial ecologists who work with microbiologists and so on to address complex global ecological problems.

Center scientists are also working together to develop a semester-long educational program in environmental sciences for undergraduate liberal arts students. Thanks to a grant from the Andrew W. Mellon Foundation, plans are almost completed for this exciting program, which is scheduled to begin in the fall of 1997 here at the MBL.

Architectural Dynamics in Living Cells Program

Headed by Distinguished Scientist Dr. Shinya Inoué, scientists in the Architectural Dynamics of Living Cells Program conduct research at the molecular and cellular level at the interface of anatomy and physiology. In 1995, Ms. Rieko Arimoto, an engineer from the Nikon Corporation in Japan, was a visiting investigator with the Program. While at the MBL she assisted Dr. Inoué and Mr. Robert Knudson in developing the four-dimensional light microscope (4-DLM) that Nikon is now manufacturing. This 4-DLM is a high-resolution light microscope that provides outstanding, dynamic three-dimensional views of the activities of living cells and the protein polymers found within them.

Dr. Inoué continues to be recognized for his outstanding scientific achievements. In 1995 he was awarded the Microscopy Society of America's highest honor, the Distinguished Scientist Award for the Biological Sciences.

The National Vibrating Probe Facility

The NIH-sponsored National Vibrating Probe Facility, which develops and makes available techniques for the non-invasive measurement of ion



fluxes between membranes, had another active year under the directorship of Peter J. S. Smith. These techniques have long included a wire voltage probe that measures nanovolt fields relating to net current flow across membranes of tissues and cells. Recently, an ion selective probe, originally developed to study calcium fluxes, has been available. It has also been successfully adapted to measure transmembrane potassium and hydrogen; studies determining its usefulness as a tool for measuring magnesium and chloride have also been completed.

In 1995 Smith and his colleagues obtained their first measurements using two new probes that are still under development. The oxygen probe, developed for the study of cell respiration, was used to make a first measurement of oxygen uptake by a single neuron. The biokelvin or aerial probe, designed to measure weak fields around tissue in a gaseous environment, has also been used successfully to measure electric fields around corn coleoptiles showing both photo and geotactic responses. Highlights of recent research done at the Facility include the use of the vas deferens of rat to study the acidification of the male reproductive tract by a proton-pumping ATPase; studies of the physiological functions of cation currents using isolated *Aplysia* bag cell neurons; measurements of ion fluxes in mouse pre-implantation embryos; and a report on acetylcholine-induced calcium fluxes across the sarcolemma of an echinoderm smooth muscle.

MBL Welcomes Two New Labs

Two new laboratories were established at the MBL in 1995. MBL Corporation Member and summer investigator Dr. Robert Silver joined the year-round community in early March. Prior to coming to the MBL, Silver was an Associate Professor in Cornell's Section and Department of Physiology and Director of the Center for Advanced Imaging Technology at the College of Veterinary Medicine there. He received his Ph.D. in Zoology from the University of California, Berkeley, and his B.S. in Biology from Illinois Institute of Technology in Chicago.

Silver's research interests include understanding the role of calcium ions in the regulation of cell division and neuronal signaling; the role of sensory hair cell stereocilia in vestibular function; intracellular calcium ion regulation; application of machine vision and signal processing and analysis methods and computational visualization to video imagery; biochemical and biophysical aspects of mitosis; cell and developmental biology; cell physiology; microinjection; and microscopy.

New England Medical Center hematologists Barbara and Bruce Furie were appointed Visiting Scientists at the MBL in 1995. They have set up a satellite year-round laboratory in the Lillie building, while maintaining their active research program in Boston.

One of the major themes of the Furie's Boston laboratory has been the biosynthesis of blood clotting proteins that contain the amino acid, γ -carboxyglutamic acid. The synthesis of this amino acid requires vitamin K—the only known use of that vitamin. With the discovery of this same amino acid in the toxins of cone snails (the only non-vertebrates proven to have γ -carboxyglutamic acid) the Furies began to wonder how cone snails make γ -carboxyglutamic acid and whether they too require vitamin K. They will address these questions, and determine whether the amino acid exists in other marine organisms, at their new lab at the MBL.

NASA Center

Eliezar (Lenny) Dawidowicz, Ph.D., was appointed the Administrator of the NASA-sponsored Center for Advanced Studies in the Space Life Sciences. Dawidowicz comes to the MBL from the National Institute of Diabetes & Digestive & Kidney Diseases in Bethesda, Maryland, where he served since 1993 as Director of the Metabolism & Structural Biology Research Program. Dawidowicz is responsible for coordinating the activities of the new Center, which will review and provide studies on a variety of life science areas related to space research. The Center is especially interested in the role that gravity plays in biological processes, and how to use variations in gravity as a probe to answer basic biological and biomedical questions.



Other Developments

Last August, the MBL entered into an exciting collaborative research agreement with the pharmaceutical company Pfizer Inc. to study the potential of marine microorganisms to produce new chemical compounds for treating diseases. MBL Senior Scientist Norman Wainwright is leading this new effort at the MBL. Wainwright has been charged with providing Pfizer with compounds made by marine microbes gathered in local waters. Pfizer then tests these compounds against thousands of different screens that detect antibiotic or anticancer activity. If Pfizer detects a novel compound, and if its antibiotic or anti-tumor activity is confirmed in subsequent tests, the compound may become one of the projects in Pfizer's drug development pipeline.

In October 1995, Drs. Roxanna Smolowitz of the University of Pennsylvania's Laboratory for Aquatic Animal Medicine and Pathology based at the MBL and Dale Leavitt of the Woods Hole Oceanographic Institution identified a relatively new disease in hard clams, *Mercentaria mercenaria*, cultured in local clam leases. The disease is caused by an organism called QPX (Quahog Parasite Unknown) and has only been identified twice previously in diseased clams in Canada. QPX is a protozoan parasite belonging to the phylum Labryinomorpha. It appears to invade the clam's mantle edges and gills causing debilitation and eventually death. The disease has made clam culturing uneconomical in Provincetown. Smolowitz and Leavitt are presently conducting research to monitor these and other clam flats, and to study QPX infectivity and transmission. Ultimately they hope to understand the pathogenesis of the disease and develop means of managing and preventing the disease.

Associate Scientist Alan Kuzirian and colleagues have recently determined experimentally that lead in seawater can be toxic to the sea slug, *Hermisenda*. Lead toxicity appears to inhibit normal feeding rhythms, to distinctly modify and depress movement, and to impair *Hermisenda*'s ability to learn. These studies have implications for further understanding the effects of lead exposure on children.

Summer Research

Hundreds of investigators from around the world came to the MBL this past summer to pursue independent research and to share findings and techniques with each other. Laboratory space was fully subscribed in 1995, and the Laboratory was able to offer \$142,000 in fellowships to 23 scientists whose proposals

to conduct research at the MBL successfully passed through a rigorous review process.

The summer of 1995 also saw the creation of the first formal "Cluster" of scientists at the MBL. The Cluster concept was developed to bring researchers with similar research interests and needs together in adjoining laboratories where they can share space, techniques, equipment, and ideas. The group comprising the inaugural Clam Cluster, including Drs. Robert Goldman (Northwestern University), Joan Ruderman (Harvard University), Katherine Swenson (Duke University), George Langford (Dartmouth College), Robert Palazzo (University of Kansas), and Avram Herskho (Technion-Israel Institute of Technology), worked together in the Whitman building to learn more about cell division and the cell cycle, using the surf clam *Spisula* as their model research organism.

The MBL's 1995 General Scientific Meetings were held from August 14 to 16, and were co-chaired by Robert Paul Malchow (University of Illinois at Chicago); Anne Giblin (MBL Ecosystems Center); and Kathleen Siwicki (Swarthmore College). More than 60 papers were presented at the meeting, 55 of which were reviewed and published as Short Reports in the October issue of *The Biological Bulletin*. Topics included biophysics, comparative physiology and biochemistry, behavior, physiological ecology, biogeochemistry and nutrient cycling, calcium, neurobiology, development, and the cell cycle.

Three Short Reports, co-authored by Mark Martindale, Jonathan Henry, and Barbara Boyer, were selected as feature articles by the *Bulletin's* editors. These papers on experimental embryology illustrated how new methods and techniques can be used to address classic questions in biology, and provided new information about how embryos are put together and how they work.



Education at the MBL

In 1995 the Howard Hughes Medical Institute (HHMI) awarded \$2 million to the Marine Biological Laboratory. The four-year HHMI grant will provide support to the MBL educational program, and specifically to courses in Physiology, Embryology, Neurobiology and Development of the Leech, Methods in Computational Neuroscience, Microbial Diversity, Biology of Parasitism, Neural Systems and Behavior, Neurobiology, and Molecular Evolution. In 1995, the MBL again offered these and six other outstanding courses, which enrolled 338 students representing 226 institutions and 39 countries.

1995 also marked the successful turnover of the directorships of a number of MBL courses. Gary Banker (University of Virginia) and Daniel Madison (Stanford) took the helm of the Neurobiology course; Janis Weeks (University of Oregon) and Harold Zakon (University of Texas, Austin) assumed the directorship of the Neural Systems and Behavior course; Edward Leadbetter (University of Connecticut) and Abigail Salyers (University of Illinois) became directors of the Microbial Diversity course; Celia Brosnan (Albert Einstein) and Jack Rosenbluth (New York University) assumed the directorship of the Pathogenesis course; and Pierre Drapeau (McGill University) and Marty Shankland (Harvard Medical School) led the Neurobiology of the Leech course, which will now be offered annually. On behalf of the MBL, I extend my thanks to all retiring course directors and to this new class of course faculty.

Last May an unusual group of students gathered at the MBL to take one of the Laboratory's newest short courses, "Aquaculture for Regulators." Fisheries officials from throughout southeastern Massachusetts participated in the one-week course, which was co-sponsored by the Woods Hole Oceanographic Institution and directed by Roger Hanlon and Dale Leavitt (WHOI). The course covered the history of aquaculture, the state-of-the-science in systems design and engineering, the biology and diseases of commercially important shellfish and finfish, and the laws and regulations pertaining to aquaculture.

Honors

MBL Corporation Members and course alumni continue to be recognized for their outstanding research efforts in the life sciences. MBL Embryology course alumnus Eric F. Wieschaus, a professor at Princeton University, was awarded the Nobel Prize in Physiology or Medicine last October for his groundbreaking work during the 1970s and early 1980s on the genetic control

of early embryonic development. The work for which he and his colleagues Christiane Nüsslein-Volhard (Max Planck Institute for Developmental Biology in Tuebingen) and Edward B. Lewis (California Institute of Technology) were recognized followed on the heels of Wieschaus' participation in the MBL's Embryology course. Wieschaus took the course in 1969 before beginning his graduate work.

"The biggest benefit [of the Embryology course] was seeing embryos from a wide variety of marine organisms," Wieschaus says of his MBL experience. "In the embryology course we could watch their development and try simple manipulative procedures to move cells around or destroy certain regions of the egg. I left the course with a deeper appreciation of the richness of developmental phenomena and the conviction that I could study embryos for the rest of my life."

MBL Corporation Member and Massachusetts Institute of Technology molecular biologist Alexander Rich was awarded the Medal of Science, the Nation's highest scientific honor, in October of 1995. The Society for Developmental Biology presented long-time MBL Corporation Member and former Trustee John Philip Trinkaus with its first Edwin G. Conklin Medal in Developmental Biology at the Society's annual meeting in late August. We also congratulate MBL Corporation Members Clara Franzini-Armstrong and Holger Jannasch and MBL course alumni Lawrence Gold, Judith Kimble, Ronald Sederoff, Douglas Wallace, and Robert Huber on their election in 1995 to membership in the National Academy of Sciences.

MBL/WHOI Library

The MBL/WHOI Library successfully installed its new Library software system, named MARINER, in December of 1995. This new system will allow the Library to store and provide information to its scientific customers more reliably, especially given the ever increasing electronic demands on the facility. MARINER now allows scientists to search for and retrieve the full text of many articles without ever leaving their computers. Although the Library is leaping with both feet into the electronic era, it still carefully maintains its extremely valuable paper collection. Last year funds donated in memory of Homer P. Smith sponsored the restoration of the Library's 1670 editions of the *Philosophical Transactions*. The Library's oldest and most valuable volume, Gesner's *Nomenclature* (dated 1560), was also sent out for restoration and repair.

Staffers from the MBL/WHOI Library's Information Systems Division were involved in 1995 in the

development of the Marine Animal web page that is now available over the Internet. This resource originated from the MBL's Marine Resources Catalog, which lists more than 200 organisms available from and distributed by the MBL. The ISD staff has expanded the content of the catalog to include images of animals, taxonomic information, current literature citations, access to DNA sequence data, and ordering information, making this new product a powerful and important resource for scientists working on marine biomedical models around the world.

The Library has also been collaborating with the editors of *The Biological Bulletin* to develop an electronic companion to the 99-year-old journal. The new electronic, peer-reviewed journal, the *Marine Models Electronic Record*, will publish practical information about non-mammalian marine organisms that are useful in basic biological and biomedical research. The MBL received a start-up grant from the National Science Foundation to launch the journal, which is scheduled to be published and available on-line by the time this report is published.

Facilities

In 1995 the MBL restored the Broderick House, located on the corner of North and Albatross Streets. MBL consultants and Town of Falmouth officials determined, after careful review, that the building was structurally unsuitable for renovation and that the original structure needed to be razed. The new Broderick House, which opened its doors in the fall, retains the look and the historical nature of the original building. Although the exterior of Broderick looks much like the original building, the interior has been modernized and converted into office space that is now occupied by the Boston University Marine Program administration and the new NASA program. The Instrument Development Laboratory occupies the basement of the facility.

Renovations to the Swope Center's second floor dining room and kitchen were also completed in 1995. New appliances were installed in the kitchen, the service area was redesigned, and both the private and main dining rooms underwent major facelifts. Sodexo, the

MBL's contracted food service, funded the renovations, which are the first renovations to the dining facilities since the Center was built in 1972.

MBL Trustees

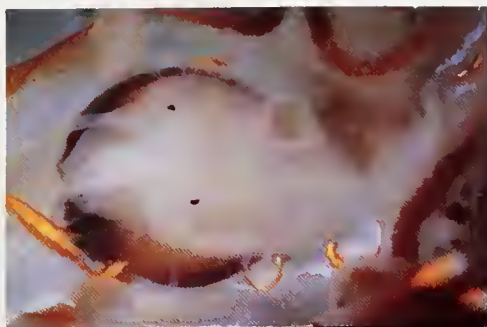
The Marine Biological Laboratory Board of Trustees elected four new members in 1995: Dr. Story Landis, Dr. Irwin Levitan, Dr. Frank Press, and Mr. Christopher Weld. All will serve as members of the Class of 2000. Dr. Landis is Scientific Director of the National Institute of Neurological Disorders and Stroke at the National Institutes of Health and Professor and Chairman of the Department of Neurosciences at Case Western Reserve University School of Medicine. Dr. Levitan is the Nancy Lurie Marks Professor of Neuroscience and Director of the Center for Complex Systems at Brandeis University. Dr. Press served two terms as president of the National Academy of Sciences (from 1981 to 1993) before being named to his present position as the Cecil and Ida Green Senior Fellow at the Carnegie Institution of Washington. Mr. Weld is Managing Partner of the Boston law firm Sullivan and Worcester.

I also extend my thanks, on behalf of the members of the Board, to retiring Board members Drs. Robert Goldman and Eric Davidson for all of their efforts during their tenure as Trustees.

Looking Ahead

Following the report of the Decennial Review Committee in 1994, the MBL's Board of Trustees, the Science Council, and various small groups of scientists and individuals have begun the process of planning for the Marine Biological Laboratory's future. These groups have spent considerable time reviewing the role of the Laboratory as a force in American biological and biomedical research and training. This process, which is on-going, will be of the utmost importance over the coming years as we seek to strengthen the Laboratory in light of the advances in knowledge and the increased sophistication and cost of biological research.

—John E. Burris



Report of the Treasurer

This report accompanies the financial statements for the Marine Biological Laboratory for the year ended December 31, 1995. Although the continuing solid financial performance and increasing financial strength of the Laboratory is the heart of my message, I should spend a little time orienting the reader to the new method of presenting financial position and results that one will confront in the pages following this report. In 1995 the Laboratory adopted the reporting format mandated by Statement of Financial Accounting Standards 117 (SFAS 117) whose purpose it is to simplify and standardize reporting of not-for-profit institutions and make their financial statements more comparable to for-profit businesses.

The essence of the change is to categorize all activity as it affects the net assets of the institution; said net assets are subdivided into three types:

(1) *Unrestricted* net assets are immediately at the MBL's disposal; the funds for normal operating activities and balances are included here.

(2) *Temporarily restricted* net assets are those that are accompanied by some granting or donating institution's limitation on the manner or the time period in which the institution may use such funds; the unexpended balance of a multi-year grant for a specific program of research would be an example of this category.

(3) *Permanently restricted* net assets represent funds that may not be spent by the MBL, although the income deriving from investment of these funds may be spent. The original corpus of permanent endowment funds would be an example of this category.

In addition, the Laboratory has adopted the reporting requirements of SFAS 116, which primarily requires that pledges be booked as an asset by the institution, even though the cash has not yet been received, just as an account receivable would be. The result of adopting this standard was a one-time increase in the net assets of the Laboratory of \$1.3 million.

The status of these net assets is presented in the Balance Sheet; activities that affected them are presented in the Statement of Activities and the way in which these activities changed the cash position of the Laboratory is presented in a new statement, the Statement of Cash Flows.

The net assets of the Marine Biological Laboratory increased in 1995 from \$42.5 million to \$48.2 million. This increase was essentially due to the success of our investment management activities; we enjoyed realized and unrealized capital gains of \$4.9 million and a total return on our portfolio for the year of 30%. This was a happy payoff to the significant work of the Finance and Investment Committee in restructuring the process of managing the Laboratory's investments. This investment success is the major cause for the increases in temporarily restricted net assets of \$5,539,000 and of permanently restricted net assets of \$857,000.

Unrestricted Net Assets of \$21,050,000 represented a decrease of \$739,818, a result after the impact of depreciation of about \$1,410,049. In accordance with SFAS 117, the Laboratory is reporting the results of operations after depreciation; this result underlines the continuing necessity to generate funds for the replacement of our scientific and other facilities, but masks a comparatively strong and healthy result of operations for the year.

Our liquidity has diminished as our current investments have dropped \$1,300,000. This decrease was due in large measure to the conclusion of a multi-year grant in support of the education program. The grant, from the Howard Hughes Medical Institute, has been renewed but the funding terms have been changed. The MBL will receive this cash annually in the future, rather than for a number of years in advance.

The major financial management event of 1995 is that it is the last full year the Laboratory was the beneficiary of the extraordinary services of John Speer, Controller. John retired in June, 1996. When John joined the Laboratory in 1982 the net assets of the MBL were \$10 million. Today they are \$48 million. During

that time there have been three directors and one acting director. A major new research facility has been built. There have been three major debt financings. An employee bargaining unit was formed and the Board of Directors restructured itself. The federal government changed its mind a number of times as to how its grant funds should be handled and those activities reported, and the Financial Accounting Standards Board has changed its reporting requirements a few more times. Shortly after he arrived at the MBL John implemented a new financial management system. Having outlived that one, John initiated another renewal of the computer system this year. All of this after John had

retired as a captain from the U.S. Navy. His energy is boundless and his ability to surface issues while there is plenty of time to consider them has served this institution well. It's hard to believe John is going to retire a second time. He will be missed.

As John leaves, the Laboratory recognizes that the position has changed; through his actions, John created the position of Chief Financial Officer of the Marine Biological Laboratory, and his replacement, Timothy Roddy, who comes to us from the National Academy of Sciences, will hold that title.

—Robert Manz

Coopers
& Lybrand

certified public accountants

REPORT OF INDEPENDENT ACCOUNTANTS

To the Board of Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have audited the accompanying balance sheet of Marine Biological Laboratory (the "Laboratory") as of December 31, 1995 and the related statements of activities and cash flows for the year then ended. We previously audited and reported upon the financial statements of the Laboratory for the year ended December 31, 1994, for which condensed statements are presented for comparative purposes only. These financial statements are the responsibility of the Laboratory's management. Our responsibility is to express an opinion on these financial statements based on our audit.

We conducted our audit in accordance with generally accepted auditing standards. Those standards require that we plan and perform the audit to obtain reasonable assurance about whether the financial statements are free of material misstatement. An audit includes examining, on a test basis, evidence supporting the amounts and disclosures in the financial statements. An audit also includes assessing the accounting principles used and significant estimates made by management, as well as evaluating the overall financial statement presentation. We believe that our audit provides a reasonable basis for our opinion.

In our opinion, the financial statements referred to above present fairly, in all material respects, the financial position of Marine Biological Laboratory at December 31, 1995, and its activities and cash flows for the year then ended in conformity with generally accepted accounting principles.

As discussed in Note B to the financial statements, during 1995 the Laboratory changed its method of accounting for contributions received and adopted newly established financial reporting display standards for not-for-profit organizations. The accounting for contributions was adopted prospectively and the financial reporting display was adopted retroactively and the summarized comparative financial information presented for the year ended December 31, 1994 has been restated.

Our audit was conducted for the purpose of forming an opinion on the basic financial statements taken as a whole. The supplemental schedules for unrestricted net assets (Schedule I), temporarily restricted net assets (Schedule II), permanently restricted net assets (Schedule III), and pooled investments (Schedule IV) as of December 31, 1995 are presented for purposes of additional analysis and are not a required part of the basic financial statements. Such information has been subjected to the auditing procedures applied in the audit of the basic financial statements and, in our opinion, is fairly stated, in all material respects, in relation to the basic financial statements taken as a whole.

Boston, Massachusetts
May 3, 1996

Coopers & Lybrand

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1995

(with comparative totals for 1994)

	ASSETS	1995	1994	LIABILITIES AND FUND BALANCES	1995	1994
Cash		\$ 844,528	\$ 589,202	Current portion of long-term debt (Note F)	\$ 76,670	\$ 79,010
Investments, at market (Note C)		1,552,459	2,864,732	Current obligations held under capital leases (Note E)	33,101	28,014
Accounts receivable, net of allowance for doubtful accounts of \$10,000 in 1995 and 1994		787,128	809,216	Accounts payable and accrued expenses	1,945,977	1,811,037
Pledge receivable, net of allowance for doubtful pledges of \$5,345 (Note H)		2,461,517	—	Deferred income	239,145	246,746
Receivables due for costs incurred on grants and contracts		1,616,678	1,014,323	Advances on contracts	195,467	21,110
Other assets		467,052	455,008	Total current liabilities	2,490,360	2,185,917
Total current assets		7,729,362	5,732,481	Annuities and unitrusts payable (Note B)	1,076,320	785,096
Investments, at market (Notes C and D)		25,365,676	20,115,871	Long-term debt (Note F)	2,320,000	2,396,671
Plant assets (Notes B and F):				Obligations held under capital leases (Note E)	29,470	44,687
Land		689,661	689,661	Advances on contracts	400,000	—
Buildings		31,730,830	31,138,144	Total long-term liabilities	3,825,790	3,226,454
Equipment		3,496,593	3,316,335	Commitments and contingencies (Note E)		
Construction in progress		—	49,387	Total liabilities	6,316,150	5,412,371
Less accumulated depreciation		35,917,084	35,193,527	Net assets:		
Plant assets, net		(14,534,514)	(13,124,465)	Unrestricted (Note B)	20,310,276	21,050,094
Total long-term assets		21,382,570	22,069,062	Temporarily restricted (Note B)	15,882,483	10,343,075
		46,748,246	42,184,933	Permanently restricted (Note B)	11,968,699	11,111,874
Total assets		\$54,477,608	\$47,917,414	Total net assets (Note I)	48,161,458	42,505,043
				Total liabilities and net assets	\$54,477,608	\$47,917,414

The accompanying notes are an integral part of the financial statements.

Marine Biological Laboratory

Notes to Financial Statements

A. Purpose of the Laboratory:

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

B. Significant Accounting Policies:

Basis of Presentation

The accompanying financial statements have been prepared on the accrual basis and in accordance with the principles of not-for-profit accounting.

The Laboratory has adopted Statement of Financial Accounting Standards No. 116, Accounting for Contributions Received and Made which requires the recording of certain promises to give (gift pledges) and other contributed services. The provisions of FAS 116 have been adopted prospectively, and accordingly, a cumulative adjustment for change in accounting principles is reflected in these statements.

The Laboratory has also adopted Statement of Financial Accounting Standards No. 117, Financial Statements of Not-for-Profit Organizations which requires the inclusion of a statement of cash flows and the classification of contributed net assets as permanently restricted, temporarily restricted, or unrestricted, determined by the existence or absence of restrictions placed on their use by donors. The provisions of SFAS No. 117 have been adopted retroactively, and, accordingly, the 1994 comparative totals have been restated to conform to the 1995 presentation. A description of the three net asset categories follows:

Net Assets

The Laboratory's net assets are segregated into three groups:

Unrestricted—Unrestricted net assets represent the results of operations, assets released from restrictions, and all resources not subject to donor-imposed restrictions of a more specific nature than the furtherance of the Laboratory's mission. Included in unrestricted net assets are the funds related to the \$21,382,570 of net plant assets, offset by debt and other liabilities.

Temporarily restricted—These assets include gifts plus monies for which the specific, donor-imposed restrictions have not been met and pledges, annuities, and life income trusts for which the ultimate purpose of the proceeds is not permanently restricted. As the restrictions are met, the assets are released to unrestricted. Also, gains/losses associated with permanently restricted gifts which have no donor restrictions on the realized and unrealized gains are classified as temporarily restricted but maintain the donor requirements for expenditure.

Permanently restricted—These assets include gifts, pledges and trusts which require that the corpus be invested in perpetuity and only the income be made available for program operations in accordance with donor restrictions.

Cash and Cash Equivalents

Cash equivalents consist of resources invested in overnight repurchase agreements and highly liquid investments with an original maturity of three months or less.

Investments

Investments purchased by the Laboratory are carried at market value. Donated investments are recorded at fair market value at the date of the gift. For determination of gain or loss upon disposal of investments, cost is determined based on the first-in, first-out method.

In 1924, the Laboratory became the beneficiary of certain investments, classified as permanently restricted net assets, which are held in trust by others. The Laboratory has the continuing rights to the income produced by these funds in perpetuity, subject to the contractual restrictions on the use of such funds. Accordingly, the trust has established a process to conduct a review every 10 years by an independent committee to ensure the Laboratory continues to perform valuable services in biological research in accordance with the restrictions placed on the funds by the agreement. The committee met in 1994 and determined that MBL has continued to meet the contractual requirements. The market values of such investments are \$5,457,579 and \$4,595,615 at December 31, 1995 and 1994, respectively. The income on these investments totaled \$186,505 and \$193,359 in 1995 and 1994, respectively.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

For the master pooled investments, the Laboratory employs a total return utilization policy that establishes the amount of the investment return made available for spending each year. The Finance and Investment Committee has approved the policy that the withdrawal will be based on 4% to 5% of the latest three-year ending market values of the funds. The market value includes the principal plus reinvested income, realized and unrealized gains and losses. Spending rates in excess of 5% but not exceeding 7%, must be approved in advance by the Finance and Investment Committee of the Trustees. In 1995, the Laboratory expended 4% of the year-end market value of the investments.

The net appreciation on permanently and temporarily restricted net assets is reported together with temporarily restricted net assets until such time as all or a portion of the appreciation is distributed for spending in accordance with the total return utilization policy and applicable state law.

Investment income on the pooled investment account is allocated to the participating funds on the market value unit basis (Note D).

Plant Assets

Buildings and equipment are recorded at cost. Donated facility assets are recorded at fair market value at the date of the gift. Depreciation is computed using the straight-line method, beginning the month after the asset is placed in service, over the asset's estimated useful life. Estimated useful lives are generally three to ten years for equipment and 20 to 40 years for buildings and improvements. Depreciation expense for 1995 amounted to \$1,410,049 and has been recorded in the statement of activities in the appropriate functionalized categories. When assets are sold or retired, the cost and accumulated depreciation are removed from the accounts and any resulting gain or loss is included in unrestricted income for the period.

Annuities and Unitrusts Payable

Amounts due to donors in connection with gift annuities and unitrusts are determined based on remainder value calculations, as of December 31, 1995 with varied assumptions of rates of return and payout terms.

Use of Estimates

The preparation of financial statements in conformity with generally accepted accounting principles requires management to make estimates and assumptions that affect the reported amounts of assets and liabilities and disclosure of contingent assets and liabilities at the date of financial statements and the reported amounts of revenues and expenses during the reporting period. Actual results could differ from those estimates.

Tax-Exempt Status

The Laboratory is exempt from federal income tax under Section 501(c)(3) of the Internal Revenue Code.

Professional Standards

In November 1995, the Financial Accounting Standards Board (FASB) released Statement No. 124, Accounting for Certain Investments Held by Not-for-Profit Organizations, which applies for fiscal years beginning after December 15, 1995. The statement requires that investments in equity securities and debt securities be reported at fair value with gains and losses included in a statement of activities plus certain disclosures about investments. The Laboratory will adopt this standard fiscal year 1996 which begins January 1. This standard will not materially impact the financial position or results of operations of the Laboratory as investments are already stated at market but may require additional disclosure concerning investments.

C. Investments

The following is a summary of the cost and market value of investments at December 31, 1995 and 1994:

	<i>Market</i>		<i>Cost</i>	
	<i>1995</i>	<i>1994</i>	<i>1995</i>	<i>1994</i>
Certificates of deposit	\$ 52,459	\$ 50,173	\$ 52,459	\$ 50,173
Money market securities	1,530,048	1,877,731	1,530,048	1,877,731
U.S. Government Securities	1,206,863	1,057,616	998,829	1,047,615
Corporate fixed income	3,342,219	12,248,905	3,197,937	12,598,467
Common stocks	2,770,153	7,732,931	2,202,312	7,428,618
Mutual funds	14,305,946	—	12,183,166	—
Limited partnerships	3,697,200	—	3,028,464	—
Real estate	13,247	13,247	13,247	13,247
Total investments	<u>\$26,918,135</u>	<u>\$22,980,603</u>	<u>\$23,206,462</u>	<u>\$23,015,851</u>

Investment portfolios for the years ended December 31, 1995 and 1994 are as follows:

Current Investments

Certificates of deposit	\$ 52,459	\$ 50,173	\$ 52,459	\$ 50,173
Money market securities	500,000	1,000,000	500,000	1,000,000
Mutual funds	1,000,000	1,019,462	1,004,309	1,041,641
Instruction portfolio	—	795,097	—	886,250
Total	<u>\$1,552,459</u>	<u>\$2,864,732</u>	<u>\$1,556,768</u>	<u>\$2,978,064</u>

Long-Term Investments

Pooled investments:				
Master pooled investments	18,231,994	9,782,000	15,493,704	9,949,252
Separately invested:				
General Chemical trust	4,311,663	3,636,845	3,736,796	3,445,228
Library Chemical trust	1,145,916	958,770	1,004,351	923,305
Ecosystem investments	—	4,388,463	—	4,363,838
Annuity investments	1,662,856	1,336,546	1,401,596	1,342,917
Real estate	13,247	13,247	13,247	13,247
Total	<u>25,365,676</u>	<u>20,115,871</u>	<u>21,649,694</u>	<u>20,037,787</u>
Total investments	<u>\$26,918,135</u>	<u>\$22,980,603</u>	<u>\$23,206,462</u>	<u>\$23,015,851</u>

D. Accounting for Pooled Investments:

Certain net assets are pooled for investment purposes. Investment income from the pooled investment account is allocated on the market value unit basis, and each fund subscribes to or disposes of units on the basis of the market value per unit at the beginning of the calendar quarter within which the transaction takes place. Ecosystem funds were added to the pooled funds in 1995 as temporarily restricted. The unit participation of the funds at December 31, 1995 and 1994 is as follows:

	<u>1995</u>	<u>1994</u>
Unrestricted	4,495	4,342
Temporarily restricted	43,242	8,773
Permanently restricted, restricted income	63,766	63,524
Permanently unrestricted, unrestricted income	229	229
	<u>111,732</u>	<u>76,868</u>

Pooled investment activity on a per-unit basis was as follows:

	<u>1995</u>	<u>1994</u>
Unit value at beginning of year	\$127.43	\$137.18
Unit value at end of year	159.37	127.43
Increase (decrease) in realized and unrealized appreciation	31.94	(9.75)
Net income earned on pooled investments	0.11	5.53
Total return on pooled investments	<u>\$ 32.05</u>	<u>\$ (4.22)</u>

E. Commitment and Contingencies:Capital Leases

As of December 31, 1995 the Laboratory had capital leases for office equipment. Interest rates on the obligations are between 1.55% and 6.63%. The future minimum lease payments as of December 31, 1995 are as follows:

1996	\$33,101
1997	20,770
1998	8,700
	<u>\$62,571</u>

F. Long-Term Debt:

Long-term debt at December 31, 1995 amounted to \$2,396,670. The aggregate amount of principal due for each of the next five fiscal years and thereafter is as follows:

1996	\$ 76,670
1997	80,000
1998	85,000
1999	90,000
2000	105,000
Thereafter	<u>1,960,000</u>
	2,396,670
Less current portion	(76,670)
Total	<u>\$2,320,000</u>

In 1992, the Laboratory issued \$1,100,000 Massachusetts Industrial Finance Authority (MIFA) Series 1992A Bonds and \$1,500,000 MIFA Series 1992B. These bonds pay varying annual interest rates ranging from 3.48% to 6.63%. Interest expense on this debt totaled \$156,640 for the year ended December 31, 1995. The Series 1992 A and B Bonds mature on December 1, 2012 and are collateralized by a first mortgage on certain Laboratory property.

The agreements related to these Bonds subject the Laboratory to certain covenants and restrictions. Under the most restrictive covenant of this debt, the Laboratory's operating surplus (before transfers), interest, expense and transfers from the quasi-endowment for debt service must equal or exceed all debt service payments, as defined by the agreement. The Laboratory was in compliance with these covenants and restrictions at December 31, 1995.

G. Retirement Plan:

The Laboratory participates in the defined contribution pension plan of TIAA-CREF (the "Plan"). The Plan is available to permanent employees that have completed two years of service. Under the Plan, the Laboratory contributes 10% of total compensation for each participant. Contributions amounted to \$571,285 in 1995 and \$525,918 in 1994.

H. Pledges.

Unconditional promises are included in the financial statements as pledges receivable and revenue of the appropriate net asset category. Pledges are recorded after discounting to the present value of the future cash flows.

Unconditional promises are expected to be realized in the following periods:

In one year or less	\$1,009,715
Between one year and five years	1,669,700
	<u>2,679,415</u>
Less: discount of \$212,553 and allowance of \$5,345	(217,898)
	<u>\$2,461,517</u>

Pledges receivable at December 31, 1995 have the following restrictions:

Research and education	\$2,456,917
Permanently restricted net assets	4,600
	<u>\$2,461,517</u>

I. Reconciliation of Prior Year Fund Balances.

The following is a reconciliation of total December 31, 1994 fund balances as previously reported to the restated net asset balances for the same period:

December 31, 1994 reported total fund balances	\$39,534,683
Reclassified deferred support to:	
Temporarily restricted net assets	2,675,676
Unrestricted net assets	294,684
	<u>2,970,360</u>
December 31, 1994 restated total net assets	<u>\$42,505,043</u>

J. Postretirement Benefits.

On November 20, 1993, the Laboratory adopted Statement No. 106, "Employers' Accounting for Postretirement Benefits Other Than Pensions," for the year beginning January 1, 1994. Prior to 1995, the Laboratory recognized these benefits as an expense when paid. This new standard requires employers to accrue, during the years that the employee renders the necessary service, the expected cost of benefits to be provided during retirement. As permitted, the Laboratory has elected to amortize the transition obligation over 20 years.

The Laboratory's policy is that all current retirees and certain eligible employees who retired prior to June 1, 1994 will continue to receive postretirement health benefits. The remaining current employees will receive benefits; however, those benefits will be limited as defined by the Plan. Employees hired on or after January 1, 1995 will not be eligible to participate in the postretirement medical benefit plan.

Net postretirement benefits for 1995 and 1994 include:

	<u>1995</u>	<u>1994</u>
Service cost (benefits earned during period)	\$ 61,851	\$ 54,494
Interest cost (on projected benefit obligation)	141,218	135,459
Actual return on plan assets	(13,801)	(3,032)
Net amortization and deferral	<u>84,953</u>	<u>86,918</u>
Net postretirement benefits cost	<u>\$ 274,221</u>	<u>\$ 273,839</u>

Below is a reconciliation of the funded status of the Plan at December 31, 1995 and 1994:

1995 accumulated postretirement benefit obligation:		
Retirees and dependents	\$1,435,418	\$1,195,739
Fully eligible active participants	225,602	242,279
Other active participants	<u>557,950</u>	<u>401,951</u>
Total	2,218,970	1,839,969
Market value of plan assets	<u>398,785</u>	<u>190,601</u>
Assets less than obligations	1,820,185	1,649,368
Unrecognized prior service cost (credit)	—	—
Unrecognized net (gain) loss	257,322	96
Unrecognized transition obligation	<u>1,562,803</u>	<u>1,649,625</u>
Prepaid postretirement benefit cost	<u>\$ (60)</u>	<u>\$ 161</u>

The health care cost trend rate assumptions used in determining the projected benefit obligation begins at 10.0% in 1995 and gradually decreases to 5.0% in the year 2006 and thereafter. The effect of raising the assumed health care cost trend rate by one percentage point in each year would be to increase the accumulated postretirement benefit obligation as of December 31, 1995 by \$200,027 and to increase the aggregate of the service and interest cost components of net periodic postretirement benefit cost for the year then ended by \$17,533. The discount rate used in determining the accumulated postretirement benefit obligation is 7.0%, and the expected return on plan assets was 8.0%. During 1995, the Laboratory contributed \$274,000 to fund the Trust for these postretirement benefits.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF ACTIVITIES

for the year ended December 31, 1995

	Unrestricted Assets	Temporarily Restricted Assets	Permanently Restricted Assets	1995 Total	1994 Total
Support and Revenues:					
Grant reimbursement direct costs	\$ 6,411,019			\$ 6,411,019	\$ 5,388,844
Grant for capital additions				—	185,087
Recovery of indirect costs	4,090,295			4,090,295	3,700,412
Tuition	606,460			606,460	577,365
Fees for services	3,987,004			3,987,004	3,551,692
Gifts	681,557	\$ 635,789	\$ 51,415	1,368,761	2,599,127
Pledges		2,422,025		2,422,025	—
Investment income	91,186	667,499		758,685	972,589
Miscellaneous revenue	570,210	25,266		595,436	775,731
Total support and revenues	16,437,731	3,750,539	51,415	20,239,685	17,750,847
Net assets released from restrictions	3,238,165	(3,238,165)		—	—
Present value adjustment to annuities		(225,789)	(31,759)	(257,548)	36,398
Total	19,675,896	286,585	19,656	19,982,137	17,787,245
Expenses (including \$1,410,049 of depreciation expense):					
Research	7,862,052			7,862,052	7,060,508
Instruction	2,353,740			2,353,740	1,977,933
Scholar, fellow, stipends	462,006			462,006	465,824
Services	6,077,359			6,077,359	5,641,913
Administration	3,631,694			3,631,694	3,198,945
Other	186,457			186,457	261,908
Total expenses	20,573,308			20,573,308	18,607,031
Excess (deficit) of support and revenue over expenses before gain on investments	(897,412)	286,585	19,656	(591,171)	(819,786)
Net realized gains (losses) on investments	32,442	921,208	372,614	1,326,264	1,529,800
Net unrealized gains (losses) on investments	117,152	3,033,723	459,955	3,610,830	(2,957,830)
Change in net assets	(747,818)	4,241,516	852,225	4,345,923	(2,247,816)
Cumulative adjustment for pledges	8,000	1,297,892	4,600	1,310,492	—
Net assets, beginning of year	21,050,094	10,343,075	11,111,874	42,505,043	44,752,859
Net assets, end of year	\$20,310,276	\$15,882,483	\$11,968,699	\$48,161,458	\$42,505,043

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF CASH FLOWS

for the year ended December 31, 1995

	1995	1994
Cash flows from operating activities:		
Change in net assets	\$ 4,345,923	\$ (2,247,816)
Adjustments to reconcile change in net assets to net cash (used by) from operating activities:		
Depreciation	1,410,049	1,240,197
Unrealized (gain) loss on investments	(3,610,830)	2,957,830
Realized (gain) loss on investments	(1,326,264)	(1,529,800)
Present value adjustment to annuities payable	257,548	(36,398)
Contributions restricted for long-term investment and annuities	(73,884)	(839,515)
Change in certain balance sheet accounts:		
Accounts receivable	22,088	(4,261)
Pledges receivable	(2,461,517)	0
Grants and contracts receivable	(602,355)	(108,312)
Other assets	(12,045)	(33,616)
Deposits with trustees	0	492,852
Increase (decrease) in:		
Accounts payable and accrued expenses	134,940	103,841
Deferred income	(7,601)	6,074
Advances on contracts	574,357	(18,607)
Net cash used in operating activities	<u>(1,349,591)</u>	<u>(17,531)</u>
Cash flows from investing activities:		
Purchase of property and equipment	(709,122)	(830,625)
Disposals of property and equipment	4,500	0
Proceeds from sale of investments	42,882,597	17,111,739
Purchase of investments	<u>(40,515,256)</u>	<u>(17,003,320)</u>
Net cash provided by (used in) investing activities	<u>1,662,719</u>	<u>(722,206)</u>
Cash flows from financing activities:		
Payments on annuities payable	(23,609)	(12,918)
Receipt of permanently restricted gifts	51,415	175,380
Annuity donations received	22,469	664,135
Payments on long-term debt	(79,011)	(69,009)
Payments on capital leases	<u>(29,066)</u>	<u>(18,168)</u>
Net cash (used in) provided by financing activities	<u>(57,802)</u>	<u>739,420</u>
Net increase (decrease) in cash and cash equivalents	255,326	(317)
Cash and cash equivalents at beginning of year	<u>589,202</u>	<u>589,519</u>
Cash and cash equivalents at end of year	<u><u>\$844,528</u></u>	<u><u>\$589,202</u></u>

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY
SUPPLEMENTAL SCHEDULE—UNRESTRICTED NET ASSETS

	Sponsored Research Programs	Educational Programs	Operations Support Services	Internally Designated Support Programs	Internally Designated Facilities Support	Land Buildings Equipment Assets	Unrestricted Quasi- Endowment	Total Unrestricted Net Assets
Support and Revenues:								
Grant reimbursements of direct costs	\$5,640,500	\$770,519						\$ 6,411,019
Recovery of indirect costs			\$4,090,295					4,090,295
Tuition		606,460						606,460
Fees for services:								
Dormitories			1,176,014					1,176,014
Dining hall			1,005,515					1,005,515
Library			551,423					551,423
Scientific journals		186,248	218,035					404,283
Research services			648,782					648,782
Aquatic resources			200,987					200,987
								3,987,004
Gifts			580,600	\$ 37,957	\$ 63,000			681,557
Investment income		2,339	75,520				\$ 13,327	91,186
Miscellaneous revenue		70,397	87,316	206,813	205,684			570,210
Total support and revenue	5,640,500	1,635,963	8,634,487	244,770	268,684		13,327	16,437,731
Net assets released from restrictions	1,038,093	813,731	1,377,004		9,337			3,238,165
	6,678,593	2,449,694	2,240,491	244,770	278,021		13,327	19,675,896
Expenses								
Research	6,613,827							6,613,827
Instruction		2,129,491						2,129,491
Scholarships, fellowships and stipends	113,627	186,330		25,000				324,957
Services								
Dormitories			853,407	9,332				862,729
Dining hall			979,582	3,190				982,772
Library			940,153	1,064				941,217
Scientific journals		186,248	205,006					391,254
Research services			742,417	3,227				754,664
Aquatic resources			451,214					451,214
								4,374,830
Administration (excluding depreciation and facilities operations):								
General operations			\$2,414,531		100,000			2,514,531
Research & education			540,279					540,279
Facilities operations			2,212,425	2,215	264,247			2,478,887
Depreciation			24,565			1,385,484		1,410,049
Other				186,457				186,457
Total expenses	6,727,454	2,502,069	9,363,579	230,475	364,247	1,385,484		20,573,308
Excess (deficit) of support	(48,861)	(52,375)	647,912	14,295	(86,226)	(1,385,484)	\$ 13,327	(897,412)
Realized gains on investments							32,442	32,442
Unrealized gains on investments							117,152	117,152
Transfers within unrestricted								
Debt service			(79,010)			79,010		
Acquisition of fixed assets			(96,135)		(612,987)	709,122		
Repairs and replacement			(374,000)		374,000			
Other	2,788	(11,503)	17,759	(9,142)			96	
Total transfers within unrestricted	2,788	(11,503)	(531,386)	(9,142)	(238,987)	788,132	98	
Net changes in unrestricted net assets	(46,073)	(63,878)	116,526	5,153	(325,213)	(597,352)	163,019	(747,818)
Cumulative adjustment for pledges			8,000					8,000
Net unrestricted assets, beginning of year	(16,576)	132,184	(73,255)	161,922	790,430	19,502,010	553,379	21,050,094
Net unrestricted assets, end of year	\$ (62,649)	\$ 68,306	\$ 51,271	\$ 167,075	\$ 465,217	\$ 18,904,658	\$ 716,398	\$20,310,276

MARINE BIOLOGICAL LABORATORY
SUPPLEMENTAL SCHEDULE
TEMPORARILY RESTRICTED NET ASSETS

December 31, 1995

	Donor Restricted Gifts	Annuities and Unitrusts	Pledges	Restricted Funds Treated as Quasi Endowment	Income and Gains on Permanently Restricted	Total Temporarily Restricted
Support and revenues:						
Grant reimbursement direct costs						
Grant for capital additions						
Recovery of indirect costs						
Tuition						
Fees for services						
Gifts	\$ 613,145	\$ 22,469		\$ 175		\$ 635,789
Pledges	83,948	18,077	\$ 2,422,025	159,165	406,309	2,422,025
Investment income	25,226					667,499
Miscellaneous income						25,226
Total support and revenues	722,319	40,546	2,422,025	159,340	406,309	3,750,539
Net assets released from restrictions	(2,327,284)	(25,012)		(387,836)	(498,033)	(3,238,165)
Cash received for pledges	1,263,000	(225,789)	(1,263,000)			0
Present value adjustment to annuities						(225,789)
Total	(1,604,965)	(210,255)	1,159,025	(228,496)	(91,724)	286,585
Net realized gains (losses) on investments	33,906	1,803		415,733	469,766	921,208
Net unrealized gains (losses) on investments		230,837		1,054,903	1,747,983	3,033,723
Net change in assets	(308,059)	22,385	1,159,025	1,242,140	2,126,025	4,241,516
Cumulative adjustment for pledges			1,297,892			1,297,892
Net assets, beginning of year	2,675,576	472,535		5,506,459	1,688,445	10,343,075
Net assets, end of year	\$ 2,367,517	\$ 494,980	\$ 2,456,917	\$ 6,748,599	\$ 3,814,470	\$ 15,882,483

MARINE BIOLOGICAL LABORATORY

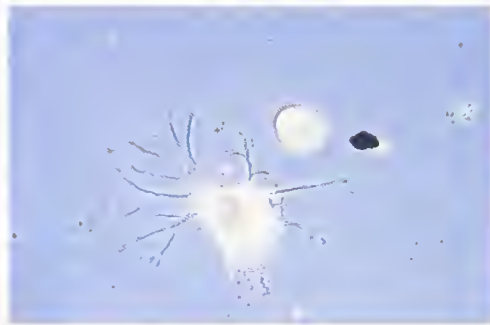
SUPPLEMENTAL SCHEDULE
PERMANENTLY RESTRICTED NET ASSETS

December 31, 1995

	<u>Annuities and Unitrusts</u>	<u>Endowment Unrestricted Income</u>	<u>Endowment Restricted Income</u>	<u>Pledges</u>	<u>Total Permanently Restricted</u>
SUPPORT AND REVENUES					
Grant reimbursement direct costs					
Grant for capital additions					
Recovery of indirect costs					
Tuition					
Fees for services					
Gifts		\$ 10,000	\$ 41,415		\$ 51,415
Pledges					
Investment Income					
Miscellaneous					
Total support and revenues					51,415
Net assets released from restrictions					
Present value adjustment to annuities	<u>\$(31,759)</u>				<u>(31,759)</u>
Total	(31,759)	10,000	41,415		19,656
Net realized gains (losses) on investments		291,568	81,046		372,614
Net unrealized (gains) losses on investments		<u>353,855</u>	<u>106,100</u>		<u>459,955</u>
Net change in assets	<u>(31,759)</u>	<u>655,432</u>	<u>228,561</u>		<u>852,225</u>
Cumulative adjustment for pledges				<u>\$4,600</u>	<u>4,600</u>
Net assets, beginning of year	<u>78,915</u>	<u>3,656,845</u>	<u>7,376,114</u>		<u>11,111,874</u>
Net assets, end of year	<u>\$ 47,156</u>	<u>\$ 4,312,268</u>	<u>\$ 7,604,675</u>	<u>\$4,600</u>	<u>\$11,968,699</u>

MARINE BIOLOGICAL LABORATORY
SUPPLEMENTAL SCHEDULE
NET ASSETS FOR POOLED INVESTMENTS AND TRUSTS
December 31, 1995

	Unrestricted	Temporarily Restricted			Permanently Restricted		
	Income Unrestricted Purposes	Income for Restricted Purposes	Income and Gains on Permanently Restricted	Total Temporarily Restricted	Income for Unrestricted Purposes	Income for Restricted Purposes	Total Permanently Restricted
SUPPORT AND REVENUES							
Gifts		\$ 175		\$ 175	\$ 10,000	\$ 41,415	\$ 51,415
Interest/dividend income	\$ 13,327	159,165	\$ 406,309	565,474			\$ 51,590
Net realized gain (loss) on investments	32,442	415,733	469,766	885,499	291,568	81,046	578,801
Net unrealized gains (loss) on investments	117,152	1,054,903	1,747,983	2,802,886	353,855	106,100	1,290,555
Total return of investments	162,921	1,629,801	2,624,058	4,253,859	645,423	187,146	3,379,993
Total support and revenues	162,921	1,629,976	2,624,058	4,254,034	655,423	228,561	5,249,349
Capitalization of income	22,125						5,300,939
Withdrawals in accordance with spending rate	(22,027)	(244,836)	(498,033)	(742,869)			22,125
Ecosystem year-end withdrawals		(143,000)		(143,000)			(764,896)
							(143,000)
Total expenses and transfers	98	(387,836)	(498,033)	(885,869)			(885,771)
Net change in assets	163,019	1,242,140	2,126,025	3,368,165	655,423	228,561	4,415,168
Net assets, beginning of year	553,379	5,506,459	1,688,445	7,194,864	3,656,845	7,376,114	18,781,202
Net assets, end of year	\$ 716,398	\$6,748,599	\$ 3,814,470	\$10,563,029	\$ 4,312,268	\$7,604,675	\$23,196,370



Report of the Librarian

With the on-going revolution in electronic publishing and delivery of information, the MBL/WHOI Library has recently been evaluating its existing electronic infrastructure. The Library's physical network and its connections to the Internet have proven to be reliable, steady, and stable. The only weak link turned out to be the Library's on-line catalog, which operated through an arrangement with a local library consortium known as "CLAMS." The software residing on the CLAMS system was not robust enough to handle some of the new demands that the MBL/WHOI Library expects with the shift to delivery of electronic journals.

As a result, the Library staff conducted a thorough search for a new software system that could handle the cross-platform computers in the community and allow logins from anyone on the Internet using a World Wide Web Browser. A new Library system, named "MARINER," was selected and installed in December of 1995. The system went on-line January 1, 1996. This new system will provide access to the bibliographic records of the Library's print collection and to the full text of some of the electronic journals to which the Library currently subscribes.

Journals

Last year the scientific community was again stunned by escalating print journal prices. In 1995 the Library was forced to cancel its subscriptions to important print journals for purely economic reasons. Part of the problem is that publishers are still vacillating between the worlds of print and electronic publications, but the number of electronic publications is increasing. In 1994 no peer-reviewed science, medical, or technology journals were published electronically, while in 1995 the number of such journals published electronically grew to 100. Information from a number of major publishers, including Elsevier Science, Springer-Verlag, John Wiley & Sons, Blackwell Science Limited and Academic Press, shows that more than 2,000 journals

are expected to be published electronically in 1996. Most publishers remain committed to print publications for archival purposes, but some of the newer electronic journals will only be archived on-line.

How will publishers charge for these new electronic publications? A number of pricing models now exist ranging from "free" on-line for a year, to "free" if you subscribe to the print version, to a 10% price increase if a user subscribes to both paper and print, to site licenses, etc. Although the problems with pricing trends of electronic publishing are numerous, we are confident that they will be solved in one fashion or another. Clearly, electronic publications are here to stay and their advantages in terms of rapid delivery of material, use of video, sound, hot links to other relevant articles, speed of retrieval, and the potential for on-line peer review outweigh the inherent problems associated with a developing technology.

In addition to the problem of increasing journal prices, the lack of adequate storage space for archival materials is ever present at the MBL/WHOI Library. This year the entire front stack of currently received journals was moved to accommodate another three years of growth. Soon we must decide how to deal with some lesser-used back issues. Electronic storage will also become a problem. Will publishers allow entire journals to be downloaded and stored on a campus server, or will libraries always have to retrieve articles from the NET? How long will a licensing agreement last? Will libraries be entitled to unlimited retrieval over the years or will they be eligible to retrieve only those years for which a subscription has been paid?

Policies

A number of new policies concerning Library access, use of Rare Book Room materials, book loans, and circulation were established in 1995 by members of the Library Staff and the Joint Users Committee, which is chaired by Dr. David Shepro. The major task now

facing the Joint Users Committee is the development of journal retention and addition policies in light of increasing journal prices. Now that all of our resources are available on the Internet, we are closely monitoring the effect it has on our Interlibrary Loan Department. Measures are also being taken to ensure that our staff spends the majority of its time addressing the needs of our primary scientific users.

Books

The book fair held in the Lillie lobby during the last two weeks in July attracted a number of vendors, whose donations of outstanding books bolstered our collection of monographs. Also in July, the Library held a dedication in honor of Dr. Kimball Atwood in whose memory a fund was established for the annual purchase of books, journals, and electronic publications in the field of genetics. Last year the Library hired a summer Library Science Student who cataloged the WHOI Marine Policy Center's collection of over 1,000 books, which are now available to the community through the Library's MARINER system.

The Archives, Preservation, and Rare Books Collection

Ralph M. Titcomb, appraiser of Rare Books for the Massachusetts and Rhode Island Antiquarian Booksellers Association, spent two days appraising the collection in the Rare Books Room. He offered valuable advice on restoring and preserving some of our most valuable books. He also recommended additional security for portions of the collection. The Rare Books preservation program continues to make great strides. This year funds donated in memory of Homer Smith sponsored the restoration of the 1670 editions of the *Philosophical Transactions*. The 1560 edition of Gesner's *Nomenclature* was also sent out for restoration and repair.

WHOI Branch Libraries

The document library that was most recently located in WHOI's Clark building has moved to its final home in the Data Library at McLean. The monumental task of moving, weeding out duplicate documents, installing compact shelving, and cataloging the WHOI Technical Reports was accomplished with relatively good humor in one month. The space in Clark previously used for the document library was converted into a state-of-the-art computer teaching facility that is being used by the Library staff for instruction.

Instruction

Over the year, the Library has placed increasing emphasis on both users' education and enhancing the librarian's role as systems designer in the area of

information retrieval. We've added more instruction to the Library's daily operations, making sure that users are able to perform their own unmediated searches. Lectures, demonstrations, and hands-on exercises on the Library's information resources, Medline, basic Internet skills, Hypertext Markup Language (HTML) Web page construction and higher end creation of HTML servers also have been presented to users. The National Library of Medicine also continues to offer its course in Medical Informatics, and in 1995 members of the Library staff taught courses in Medical Informatics to each of the MBL summer courses and participated in two Massachusetts state grants designed to teach public and school librarians basic search skills in the health science field.

Our efforts in systems design have met with great success, as evidenced by the number of "hits" the MBL's Home Page has received in the past few years. In 1994 the system was accessed approximately 60,000 times; in 1995 it was accessed 112,000 times; and in February of 1996 alone our site was accessed more than 80,000 times. The Marine Resource Center page is the most popular location within the MBL Home Page because it contains both scientific information and substantial image files. The Marine Resources page is also linked to other useful data files including the National Library of Medicine's Entrez database, adding to the versatility of the site.

The design, content, and accessibility of the MBL's Home Page also caught the attention of the McKinley Group's professional editorial team. The McKinley Group awarded the site four stars, the highest rating an Internet site can achieve in *Magellan*, McKinley's comprehensive Internet directory of over 1.5 million sites and 40,000 site reviews.

Although the future trends of library and information services remain unclear, we do know that the MBL/WHOI Library is supported by institutions that are funded largely by the federal government, which is in a stage of no-growth. Clearly the Library must learn to use new technologies efficiently and appropriately to leverage its efforts effectively. In keeping with this, we have made cooperative agreements with other libraries within our consortium and included Brown University in our interlibrary resource sharing agreements. We now participate in a courier service that daily delivers to our door the books and materials requested from the Boston Library consortium. Our roles as librarians will demand new ways of organizing information services. The World Wide Web revolution of the last two years is proof that users want direct access to information. The Library and its staff will certainly play a role in making sure that the information is organized and delivered in a timely and usable fashion.

—Catherine Norton

Educational Programs



Summer Courses

Biology of Parasitism (June 11 – August 12)

Director

Stephen Hajduk, University of Alabama, Birmingham

Course Faculty

Con Beckers, Yale University School of Medicine
John Boothroyd, Stanford University
Jean Feagin, Seattle Biomedical Research Institute
Peter Hotez, Yale University School of Medicine
Patricia Johnson, University of California School of Medicine, Los Angeles
Keith Joiner, Yale University School of Medicine
Phil LoVerde, State University of New York, Buffalo
Edward Niles, State University of New York
Edward Pearce, Cornell University
Phillip Scott, University of Pennsylvania
Alan Sher, National Institutes of Health
Buddy Ullman, Oregon Health Sciences University
Thomas Wynn, National Institutes of Health

Teaching Assistants

Peter Bradley, University of California, Los Angeles
Darrick Carter, Oregon Health Sciences University
Eric Denkers, National Institutes of Health
Mark Drew, Oregon Health Sciences University
Tanya Kersten, National Institutes of Health
Allen J. LeBlanc, Jr., University of Alabama, Birmingham
Keung Lee, State University of New York

Lecturers

Abul Abbas, Brigham & Women's Hospital
Norma Andrews, Yale University School of Medicine
Jay Bangs, University of Wisconsin
Stephen Beverley, Harvard University School of Medicine
Nino Campobasso, Cornell University
Frank Collins, Center for Disease Control, Atlanta
Harry Dickerson, University of Georgia
Robert Donald, University of Pennsylvania
Steve Ealick, Cornell University
Paul Englund, Johns Hopkins Medical School
Daniel Goldberg, Washington University
R. K. Grencis, University of Manchester

Stephen Hoffman, Naval Medical Research Institute
Roger Jagoda, Cornell University
Anthony James, University of California
Richard Komuniecki, University of Toledo
Susan Little, University of Georgia
John Mansfield, University of Wisconsin, Madison
Polly Matzinger, National Institutes of Health
Michael Mowatt, National Institutes of Health
Thomas Nutman, National Institutes of Health
Susan Paskewitz, University of Wisconsin
William Petri, University of Virginia
Steven Reed, Infectious Disease Research Institute
Mary Reynolds, University of Pennsylvania
Jose Ribeiro, University of Arizona
David Russell, Washington University Medical School
David Salks, National Institutes of Health
Kenneth Stuart, Seattle Biomedical Research Institute
John Swindle, University of Tennessee, Memphis
Christian Tschudi, Yale School of Medicine
Sam Turco, University of Kentucky Medical Center
C. C. Wang, University of California, San Francisco
Thomas Welles, National Institutes of Health

Course Coordinator

Robert Sabatini, University of Alabama, Birmingham

Course Assistant

Zachary Wood, University of Alabama, Birmingham

Students

Salwa F. Ahmed, Cairo University, Egypt
Andrew Brittingham, Temple University
Bridget C. Coughlin, University of Iowa
Elisa Cupolillo, Oswaldo Cruz Institute, Brazil
Cecile M. Denis, INSERM - Lillie, France
Victor Fernandez, Karolinska Institute, Stockholm
Arunava Goswami, Tata Institute of Fundamental Research, India
Ikram Guizani, Institut Pasteur de Tunis, Tunisia
Helena Helmby, Stockholm University, Sweden
Maria A. Marchetti, Yale University
Soloman S. Mpoke, Wesleyan University
Christine D. Müller-Graf, University of Oxford, England
Mary Reynolds, University of Pennsylvania
Mineko Shibayama, CINVESTAV, Mexico
Louise A. Wallace, University of Witwatersrand, South Africa
Jacqueline G. Waterkeyn, University of Melbourne, Australia

Embryology (June 13–July 26)

Directors

Eric H. Davidson, California Institute of Technology
Michael Levine, University of California, San Diego
David McClay, Duke University

Course Faculty

Marianne Bronner-Fraser, University of California, Irvine
R. Andrew Cameron, California Institute of Technology
Sean Carroll, Howard Hughes Medical Institute
Wolfgang Driever, Massachusetts General Hospital
Scott E. Fraser, California Institute of Technology
Janet Heasman-Wylie, University of Minnesota School of Medicine
Alexander D. Johnson, University of California, San Francisco
James Posakony, University of California, San Diego
Joel Rothman, University of Wisconsin
Noriyuki Satoh, Kyoto University, Japan
John Saunders, Jr., Marine Biological Laboratory
Martin Shankland, Harvard Medical School
Christopher C. Wylie, University of Minnesota School of Medicine

Teaching Assistants

Adina Bailey, University of California, San Diego
Andres Collazo, California Institute of Technology
Aaron Crawford, University of Minnesota
Mary Dickinson, Harvard University
Carmen Kirchhamer, California Institute of Technology
Catherine Krull, University of California, Irvine
Carol LaBonne, Harvard University
Catriona Logan, Duke University
Keith Maggert, University of California, San Diego
Ivan Moskowitz, University of Wisconsin, Madison
Stephan Neuhauss, Massachusetts General Hospital

Lecturers

Richard Behringer, University of Texas, Houston
Doug Melton, Harvard University
Nadia Rosenthal, Massachusetts General Hospital
Clifford Tabin, Harvard Medical School

Course Administrator

Jane Rigg, California Institute of Technology

Course Coordinator

Linda Huffer, Marine Biological Laboratory

Course Assistants

Tara Bennett, Dartmouth College
Jennie Halfant, University of Virginia

Students

Eugenio J. Carpizo-Ituarte, University of Hawaii
Simona Casarosa, University of Pisa, Italy
Demetra G. Dalamagas, Carnegie Mellon University
Francesco di Blasi, IBS-CNR, Italy
Pedro F. Fernandez Funez, Universidad Autonoma de Madrid, Spain
Steven B. Gendreau, University of Wisconsin, Madison
Andrew F. Giusti, University of California, Santa Barbara

F. James King, Duke University Medical Center
Kevin V. King, University of Missouri, Columbia
Takehiro Kusakabe, University of California, Davis
Vered Levy, Hebrew University of Jerusalem, Israel
Bin Lu, University of California, Los Angeles
Andrea R. Morris, Princeton University
Yuki Nakatani, Tokyo Institute of Technology, Japan
Nanette M. Nascone, Harvard Medical School
Tatjana Piotrowski, Max-Planck-Institute Tübingen, Germany
Andre Pires da Silva, Max-Planck-Institute Göttingen, Germany
William B. Raich, University of Wisconsin, Madison
Denise L. Robb, University of Minnesota
Alejandro Sanchez, Carnegie Institution
Natalia Sanchez Soriano, University of Cambridge, England
Richard A. Schneider, Duke University
Julie A. Segrè, Massachusetts Institute of Technology
Shankar Srinivas, Columbia University

Microbial Diversity (June 11–July 27)

Director

Edward Leadbetter, University of Connecticut
Abigail Salyers, University of Illinois

Faculty

Joel Dore, Laboratoire de Nutrition et Sécurité Alimentaire, France
Sandra Nierzwicki-Bauer, Rensselaer Polytechnic Institute
Jörg Overmann, Universität Oldenburg, Germany

Teaching Assistants

Michael Cerio, University of Connecticut
John D'Elia, University of Illinois
Marion Cecile Leclerc, Laboratoire de Nutrition et Sécurité Alimentaire, France
Thomas Pitta, Rowland Institute for Science

Lecturers

Douglas Bartlett, Scripps Institute of Oceanography
Carl Bauer, Indiana University
Paul Baumann, University of California, Davis
Paul Blum, University of Nebraska
Colleen Cavanaugh, Harvard University
Ananda Chakrabarty, University of Illinois Medical Center
William Chesbro, University of New Hampshire
Sharon Danielson, Rensselaer Polytechnic Institute
Paul Dunlap, Woods Hole Oceanographic Institute
Stephen Farrand, University of Illinois
Susan Leschine, University of Massachusetts
Kenneth Noll, University of Connecticut
Gary Olsen, University of Illinois
Norman Pace, Indiana University
Edward Ruby, University of Southern California
Nadja Shoemaker, University of Illinois
Deborah Siegle, Texas A&M University
John Waterbury, Woods Hole Oceanographic Institute
Lily Young, Rutgers University
Alexander Zehnder, Swiss Federal Institute for Environmental Science, Switzerland

Course Coordinator

Angelica Seitz, University of Connecticut

Laboratory Assistant

Judy Whittier, University of Connecticut

Students

Charles R. Anderson, University of Minnesota
David R. Arahal, University of Sevilla, Spain
Chih-Ching Chien, University of Connecticut
Laurel D. Crosby, Michigan State University
Lars R. Damgaard, University of Aarhus, Denmark
Alison L. George, University of Wales, Cardiff, England
Jiancai He, University of Massachusetts, Amherst
Elena Maria Hilario-Andrade, University of Connecticut
Christof Holliger, EAWAG Kastanienbaum, Switzerland
Dionne L. Hoskins, University of South Carolina
Sara W. Lazar, Harvard University
Eric S. Miller, North Carolina State University
Dianne K. Newman, Massachusetts Institute of Technology
Caroline M. Plugge, Wageningen Agricultural University, The Netherlands
Joy F. Sabl, University of Washington
Christian E. Schlekot, University of South Carolina
Grazyna E. Sroga, University of Chicago
Paula D. Suárez Sanchez, Universidad Simon Bolivar, Venezuela
Costantino Vetriani, University of Rome, Italy
Davide Zannoni, University of Bologna, Italy

Neural Systems & Behavior (June 11–August 4)

Directors

Janis Weeks, University of Oregon
Harold Zakon, University of Texas

Faculty

David Bodznick, Wesleyan Hall
Ronald L. Calabrese, Emory University
Catherine Carr, University of Maryland
Patsy Dickinson, Bowdoin College
Douglas L. Falls, Emory University
W. Otto Friesen, University of Virginia
David Glanzman, University of California, Los Angeles
Masashi Kawasaki, University of Virginia
Richard Levine, University of Arizona
Christine Li, Boston University
Shawn Lockery, University of Oregon
Pierre Meyrand, University of Bordeaux, France
Michael Nusbaum, University of Pennsylvania School of Medicine
Bruce O'Gara, Barnard College
Glen Prusky, University of Lethbridge, Canada
William Roberts, University of Oregon
Erin Schuman, California Institute of Technology
Darrell R. Stokes, Emory University

Teaching Assistants

Satoshi Amagai, University of Maryland
Cecilia Armstrong, University of Oregon
Heather Cook, Emory University
Sarah Craven, Boston University
Richard Dyck, The Salk Institute
Shannon Dyck, University of Lethbridge, Canada
Jorge Golowasch, Howard Hughes Medical Institute
Chou Hung, California Institute of Technology
Richard Hyson, Florida State University

David Kantor, California Institute of Technology
David Lenzi, University of Oregon
Michael Lewicki, California Institute of Technology
Jane Lubischer, University of Oregon
Farzan Nadim, Emory University
David Sandstrom, University of Arizona
James Weimann, Stanford University
Calvin Wong, University of California, San Diego

Lecturers

Gwen Jacobs, University of California, Berkeley
Sue Kinnamon, Colorado State University
Terry Takahashi, University of Oregon
Tim Tully, Cold Spring Harbor Lab
Susan Udin, State University of New York, Buffalo

Course Coordinator

Rachel Spierling, Blair Academy

Course Assistant

Kyle Lennon, Georgia Tech

Students

Dawn M. Blitz, University of Pennsylvania
Mark R. Bower, University of Arizona
Fabrizio Gabbiani, California Institute of Technology
Christine L. Gee, Queen's University, Ontario, Canada
Warren M. Grill, Case Western Reserve University
Sherri L. Hitz, Florida State University
Carsten D. Hohnke, Massachusetts Institute of Technology
Marcia Karen, University of California, San Diego
James Kozloski, University of Pennsylvania
Christiane Linster, Harvard University
Carrie Lynn Marín Bivens, University of California, Santa Barbara
Katherine T. Moortgat, University of California, San Diego
Benjamin D. Philpot, University of Virginia
Michael L. Ramaker, University of Pennsylvania
Susan Renn, Washington University
Kathryn S. Richards, Brandeis University
Paul L. Schaefer, Case Western Reserve University
Tom V. Smulders, Cornell University
Todd W. Troyer, University of California, San Francisco
Stephanie A. White, Stanford University

Neurobiology (June 11–August 12)

Directors

Gary Banker, University of Virginia Medical School
Daniel Madison, Stanford University Medical Center

Course Faculty

Elizabeth Apel, Washington University Medical School
Mark Bennett, University of California, Berkeley
Paul Bridgman, Washington University School of Medicine
Andres Buonnano, National Institutes of Health
John Heuser, Washington University School of Medicine
Stephen Jones, Case Western Reserve University
Bechara Kachar, National Institutes of Health
Stefanie Kaech, Friedrich-Miescher Institut, Germany
Maurine Linder, Washington University School of Medicine
Diane Lipscombe, Brown University

Jorge Moreira, National Institutes of Health
 David Ogden, National Institute for Medical Research
 Thomas Reese, National Institutes of Health
 Peter Reinhart, Duke University Medical Center
 Robert Rosenberg, University of North Carolina
 Morgan Sheng, Howard Hughes Medical Institute
 Carolyn Smith, National Institutes of Health
 Mark Terasaki, University of Connecticut Health Center
 Susan Wray, National Institutes of Health

Lecturers

David Bredt, University of California, San Francisco
 John Chludzinski, National Institutes of Health
 Jonathan Cohen, Harvard Medical School
 Joseph Culotti, Mount Sinai Hospital Research Institute
 Gregory Gasic, Cell Press
 Patricia Goldman-Radic, Yale University School of Medicine
 Michael Greenberg, John F. Enders Pediatric Laboratories
 Peter Hollenbeck, Harvard University
 Daniel Jay, Harvard University
 Len Kaezmarek, Yale University School of Medicine
 Gail Mandel, State University of New York, Stony Brook
 Andrew I. Matus, Friedrich-Miescher Institut, Switzerland
 Chris Miller, Brandeis University
 Richard Mooney, Duke University Medical Center
 Jonathan Raper, University of Pennsylvania School of Medicine
 Joshua Sanes, Washington University School of Medicine
 Menahem Segal, National Institutes of Health
 Li-Huei Tsai, Harvard Medical School

Course Coordinator

Stephanie Kaeck, Friedrich-Miescher Institut, Switzerland

Course Assistant

Muffie Fulton, Brown University

Students

Mary Beth Bauer, National Institute of Environmental Health Science
 Guoqiang Bi, University of California, Berkeley
 Ruth A. Bodner, University of California, San Diego
 Pamela M. England, California Institute of Technology
 Eva M. Finney, University of California, Berkeley
 Bhagwati Gupta, Tata Institute of Fundamental Research, Bombay, India
 Phyllis I. Hanson, Yale University
 Sabine N. Hilfiker-Rothenfluh, The Rockefeller University
 Tatsumi Hirata, Nagoya University, Japan
 Melissa M. Hsu, Rutgers University
 Jon S. Poling, Georgetown University
 Naibo Yang, Thomas Jefferson University

Physiology (June 11–July 22)

Director

Mark Mooseker, Yale University

Course Faculty

James Anderson, Yale School of Medicine
 William Bement, University of Wisconsin
 Steven Block, Princeton University

Kerry Bloom, University of North Carolina
 William Busa, Johns Hopkins University
 Richard Cheney, Yale University
 Laura Davis, Duke University Medical Center
 Alan Fanning, Yale School of Medicine
 Mary Lou Guernot, Dartmouth College
 Leah Haimo, University of California, Riverside
 Clay Lyddane, not available
 C. Robertson McClung, Dartmouth College
 Robert E. Palazzo, University of Kansas
 David Rimm, Yale University School of Medicine
 Roger D. Sloboda, Dartmouth College
 Edwin Taylor, University of Chicago
 Joseph S. Wolenski, Yale University

Teaching Assistants

Mary Lynn Benka, Oregon State University
 Linda Ferrans, Johns Hopkins University
 Luis Vidali, University of Massachusetts, Amherst
 Charlie Yang, University of North Carolina, Chapel Hill
 Sam Yang, University of North Carolina, Chapel Hill
 Elaine Yeh, University of North Carolina, Chapel Hill

Lecturers

Cathy Berlot, Yale Medical School
 David Burgess, University of Pittsburgh
 Steve Byers, Georgetown University
 Tim Caspar, not available
 Susan Gilbert, University of Pittsburgh
 Elizabeth Luna, Worcester Foundation for Biomedical Research
 Paul Matsudair, Massachusetts Institute of Technology
 Kimberly Mowry, Brown University
 Trina Schroer, Johns Hopkins University
 Mary Tierney, University of Vermont
 Kristi Wharton, Brown University

Course Assistants

Caroline Day, Yale University
 Raymond Murray, Connecticut College

Students

Yama A. Abassi, University of California, Santa Barbara
 Sharon L. Achilles, Johns Hopkins University
 Karen A. Beningo, University of Michigan
 Emanuela M. Bonfoco, Karolinska Institute, Sweden
 Kristin Boylan, University of Minnesota
 Joan Cerdá, Whitney Laboratory
 Susan J. Eagle, Medical College of Georgia
 Patricia A. Elkins, Purdue University
 Michael Ezrokhi, Brown University
 Steven P. Gross, University of Texas, Austin
 Robert E. Guldberg, University of Michigan
 Rodney K. Guy, Scripps Research Institute
 Elizabeth A. Holleran, University of Pennsylvania
 Joseph E. Italiano, Florida State University
 Jerry A. Kelly, University of California, Riverside
 Mira F. Krendel, Rutgers University
 Paul C. Kuo, Stanford University
 Margot E. Leonard, University of Pennsylvania
 Wenhong Li, University of California, San Diego
 Craig A. Mandato, University of Waterloo, Canada
 Thomas M. Maynard, University of Oregon
 Coleen T. Murphy, Stanford University



Timothy N. Oliver, University of North Carolina, Chapel Hill
 Tadas Panavas, University of Massachusetts, Amherst
 Katya M. Prince, Duke University
 Yasuhiko Saito, Osaka University
 Karen L. Schmeichel, University of Utah
 Kang Shen, Duke University Medical Center
 Christine L. Tock, University of Texas Medical School, Houston
 Robert J. Vasquez, Lehigh University
 Todd A. Verrastro, Colorado State University
 Aihui Wang, Michigan State University
 Li Yan, University of Kansas Medical Center
 Ronelle J. Young, University of California, Davis
 Anne-Marie C. Yvon, University of Massachusetts
 Melanie K. Zitek, Brown University

Short Courses

Analytical & Quantitative Light Microscopy (May 11–May 19)

Directors

Greenfield Sluder, Worcester Foundation for Biomedical Research
 David Wolf, Worcester Foundation for Biomedical Research

Faculty

William B. Amos, Medical Research Council, England
 Steven Block, Princeton University
 Richard Cardullo, University of California, Riverside
 Lynne Cassimeris, Lehigh University
 Winifried Denk, AT&T Bell Laboratories
 Frederick Fay, University of Massachusetts Medical School
 Jeff Gelles, Brandeis University
 Shinya Inoué, Marine Biological Laboratory
 Daniel Jay, Harvard University
 Edward Salmon, University of North Carolina, Chapel Hill
 Randi Silver, Cornell University Medical College
 Kenneth Spring, National Institutes of Health
 Yu-li Wang, Worcester Foundation for Biomedical Research

Teaching Assistants

Christine McKinnon, Worcester Foundation for Biomedical Research
 Elizabeth Thompson, Worcester Foundation for Biomedical Research

Course Coordinator

Frederick Miller, Worcester Foundation for Biomedical Research

Students

John G. Aghajanian, Worcester Foundation for Biomedical Research
 Khaja M. Ahmed, AG Plant Biotech, India
 Kathy R. Bailey, University of California, San Francisco
 Robert Blumenthal, National Institutes of Health
 Dwight D. Bowman, Cornell University
 Andrea D. Branch, Mount Sinai Medical Center
 Elizabeth S. Browne, Worcester Foundation for Biomedical Research
 Mark D. Clymer, Rhone-Poulenc Rorer
 John S. Condeelis, Albert Einstein College of Medicine
 Kingsley Cox, Howard Hughes Medical Institute
 Xinjun He, National Institutes of Health
 Edward H. Hinchcliffe, University of Minnesota
 Aditya Kapil, University of Chicago
 Mary E. Klingensmith, Harvard Medical School
 Le Ma, Harvard Medical School
 Patricia A. Mennitt, Cornell Graduate School of Medical Sciences
 Mark T. Palfett, Los Alamos National Laboratory
 Anu Puri, National Institutes of Health
 Sid P. Ragona, TopoMetrix
 Evelyn S. Ralston, National Institutes of Health
 Catherine J. Randall, Procter & Gamble
 Pamela L. Rockwell, Rhone-Poulenc Rorer
 Sidney L. Shaw, University of North Carolina, Chapel Hill
 Vladimir A. Sirotkin, Rutgers University
 Carolyn L. Smith, National Institutes of Health
 Cynthia A. Sterkenburg, National Institutes of Health
 Timothy G. Talomie, Bristol-Myers Squibb
 Linda M. Wysocki, Monell Chemical Senses Center

Medical Informatics (May 31–June 7)

Director

Homer Warner, University of Utah School of Medicine

Faculty

Andreas Baxevanis, National Center for Biotechnology Information
 Paul Clayton, Columbia Presbyterian Medical Center
 Peter Haug, University of Utah School of Medicine
 Lawrence Kingsland, National Library of Medicine
 Donald D.A.B. Lindberg, National Library of Medicine
 Carol Newton, University of California School of Medicine,
 Los Angeles
 Rick Rodgers, National Library of Medicine
 Robert Sideli, Columbia Presbyterian Medical Center

Course Coordinator

Sylvia Jessen, University of Utah School of Medicine

Students

Frederick A. Anderson, University of Massachusetts Medical
 Center
 Kenneth G. Barron, Kaiser Permanente, Los Angeles
 Mary B. Blackwelder, Medical College of Wisconsin
 John E. Blanton, U. S. Department of State
 A. James Bothmer, Creighton University
 Anna K. Chacko, Brooke Army Medical Center
 Rafael E. de la Hoz, New York University
 Allan J. Ebbin, FHP Health Care, California
 Trudy A. Gardner, Rush-Presbyterian-St. Luke's Medical Center
 George A. Gellert, Project HOPE
 Ralph Gonzales, University of California, San Francisco
 Mark A. Graber, University of Iowa
 Ruth A. Hodges, D. C. General Hospital
 Bennett Humphrey, Medical College of Ohio
 Nikola Jurisic, University of California, Los Angeles
 Nina P. Long, The Wistar Institute
 Elaine R. Martin, University of Illinois, Chicago
 Russell C. Maulitz, Medical College of Pennsylvania
 Kevin M. McNeill, University of Arizona
 Vincent C. Notarstefano, Flushing Hospital Medical Center
 Edward A. Oppenheimer, Kaiser Permanente, Los Angeles
 Richard K. Orr, Fallon Clinic, Worcester
 Brett A. Oxberry, Temple University Medical School
 Ellen R. Schellhause, St. Joseph Medical Center
 Natalie A. Schock, University of Maryland
 Lynne K. Siemers, Washington Hospital Center
 Mark S. Smith, George Washington University Hospital
 Mary L. Swift, Howard University
 Chris D. Tzarnas, Mercy Catholic Medical Center
 George H. Underwood, Tripler Army Medical Center

Methods in Computational Neuroscience *(July 30–August 26)*

Directors

David Kleinfeld, AT&T Bell Laboratories
 David Tank, AT&T Bell Laboratories

Faculty

Lawrence Abbott, Brandeis University
 Joseph Atick, The Rockefeller University
 Robert Barlow, Syracuse University
 William Bialek, NEC Research Institute
 James Bower, California Institute of Technology
 Dennis Bray, Cambridge University, UK

Carol Colby, National Eye Institute
 Bard Ermentrout, University of Pittsburgh
 Stephen Fisher, Yale University
 David Hansel, Ecole Polytechnique, France
 Nancy Kopell, Boston University
 Terry Kovacs, AT&T Bell Laboratories
 John Lisman, Brandeis University
 Rodolfo Llinás, New York University Medical Center
 Robert Malinow, Cold Spring Harbor Laboratory
 Kevin Martin, University of North Carolina, Chapel Hill
 John H. R. Maunsell, Baylor College of Medicine
 Michael Merzenich, University of California School of Medicine
 John Rinzel, National Institutes of Health
 Edmund Rolls, University of Oxford, England
 Terrence, Sejnowski, The Salk Institute
 H. Sebastian Seung, AT&T Bell Laboratories
 Arthur Sherman, National Institutes of Health
 Karen Sigvardt, National Science Foundation
 Frederick Sigworth, Yale School of Medicine
 Haim Sompolinsky, The Hebrew University of Jerusalem, Israel
 Roger Traub, IBM
 David Van Essen, Washington University Medical School
 Michael Vanier, California Institute of Technology
 Michael Webster, University of Nevada
 John White, Boston University
 Matthew Wilson, Massachusetts Institute of Technology
 Rafael Yuste, AT&T Bell Laboratories
 Steven Zucker, McGill University, Canada

Lecturers

Barry Connors, Brown University
 Leon Cooper, Brown University
 John Hopfield, California Institute of Technology
 Stephen Kosslyn, Harvard University

Lab Instructors

Michael Hines, Yale University
 Roderick Jensen, Wesleyan University

Course Assistants

Winfried Denk, AT&T Bell Laboratories
 Jennie Halfant, University of Virginia
 Rob de Ruyter Van Steveninck, NEC Research Institute

Students

Kurt E. Ahrens, University of California, Berkeley
 Hagai Attias, Yale University
 Michael Beierlein, Brown University
 Rani Ben-Yishai, The Hebrew University of Jerusalem, Israel
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Microinjection Techniques (May 23 – May 30)

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Paolo Rinaudo, Yale University School of Medicine
Stephen L. Rogers, University of Illinois
Paul F. Silverman, Rutgers University
Yi-lu O. Yuan, Harvard University

Neurobiology & Development of the Leech ***(August 5 – August 26)***

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Susanna Blackshaw, University of Glasgow, Scotland
Peter Brodfehrer, Bryn Mawr College
Francisco Fernandez de Miguel, Universidad Nacional Autonoma, Mexico
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Mitch C. Snaders, Whitehead Institute
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Yong Wang, Purdue University

Optical Microscopy (October 25 – November 1)

Director

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Brian Herman, University of North Carolina, Chapel Hill
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Shinya Inoué, Marine Biological Laboratory
H. Ernst Keller, Carl Zeiss, Inc.
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 Toshiki Yagi, University of Tokyo, Japan
 Hal F. Yee, University of California, San Francisco

Pathogenesis of Neuroimmunologic Diseases ***(August 13–August 25)***

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 Jack Rosenbluth, New York University Medical Center

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 Robert Darnell, The Rockefeller University
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 John Griffin, Johns Hopkins University School of Medicine
 William Hickey, Dartmouth-Hitchcock Medical Center
 Gilla Kaplan, The Rockefeller University
 David Kaufman, Montefiore Medical Center
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 Anthony Reder, University of Chicago
 J. Murdoch Ritchie, Yale University School of Medicine
 Luiz Rizzo, National Eye Institute
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 Bhagwan Shahani, University of Illinois, Chicago
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 Dominique S. Tews, Mainz University Medical Center, Germany
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Workshop on Molecular Evolution ***(August 6–August 18)***

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Douglas Shanklin
University of Tennessee

Hsien-Yu Wang
SUNY/Stony Brook Medical Center

Gerald Weissmann
NYU Medical Center

Michael Zigmond
University of Pittsburgh

Spiegel, Melvin, Dartmouth College
 Spotte, Stephen, University of Connecticut
 Stephen, Michael, Rutgers University
 Sundquist, Eric, United States Geological Survey
 Sweet, Frederick, Washington University

Thiele, Dennis J., University of Michigan Medical School
 Tilney, Lewis, University of Pennsylvania
 Trager, William, MBL
 Treisman, Steven N., University of Massachusetts Medical Center
 Tweedell, Kenyon S., University of Notre Dame
 Tykocinski, Mark L., Case Western Reserve University

Van Holde, Kensal E., Oregon State University

Walton, Alan J., Cambridge University
 Warren, Leonard, Wistar University
 Webb, Maggie H., Woods Hole, MA
 Weidner, Earl, Louisiana State University
 Whittaker, J. R., University of New Brunswick
 Wilber, Charles G., Colorado State University
 Wittenberg, Beatrice, Albert Einstein College of Medicine
 Wittenberg, Jonathan, Albert Einstein College of Medicine
 Wolken, Jerome J., Carnegie Mellon University
 Woodrum, David A., Medical College of Georgia

Yevick, George J., Stevens Institute of Technology
 Yevick, Miriam L., Rutgers State University

Zapol, Warren M., Massachusetts General Hospital
 Zottoli, Steven J., Williams College

Domestic Institutions Represented

Albert Einstein College of Medicine
 American Psychological Association
 American Red Cross
 American Type Culture Collection
 Arizona, University of
 Arkansas, University of, Medical Sciences
 AT&T Bell Laboratories, Inc.

Barnard College
 Barry University
 Baylor College of Medicine
 Bell Communications Research
 Bellcore
 Beloit College
 Beth Israel Hospital
 Biotechnology Research Institute
 Blair Academy
 Boston University
 Boston University School of Medicine
 Bowdoin College
 Bowman Gray School of Medicine of Wake
 Forest University
 Brandeis University
 Brigham & Women's Hospital
 Bristol-Myers Squibb
 Brooke Army Medical Center
 Brooklyn College of the City University of
 New York
 Brown University
 Bryn Mawr College

California Institute of Technology
 California State University, Fresno
 California, University of, Berkeley
 California, University of, Davis
 California, University of, Irvine
 California, University of, La Jolla
 California, University of, Los Angeles
 California, University of, Riverside
 California, University of, San Diego
 California, University of, San Francisco
 California, University of, Santa Barbara
 California, University of, Santa Cruz
 California, University of, School of Medicine
 Cambridge NeuroScience, Inc.
 Carl Zeiss, Inc.
 Carnegie Institution
 Carnegie Mellon University
 Case Western Reserve University
 Center for Disease Control
 Center for Marine Science Research
 Chemical Industry Institute of Toxicology
 Chicago, University of
 Cincinnati, University of
 Cold Spring Harbor Laboratories
 Colorado State University
 Columbia University
 Connecticut, University of
 Connecticut, University of, Health Center
 Cornell Graduate School of Medical Sciences
 Cornell University
 Creighton University

D. C. General Hospital
 Dartmouth College
 Duke University
 Duke University Medical Center

Eckerd College
 Ecovale Research
 Emory University

Fallon Clinic
 Fitch University of Health Sciences
 FHP Health Care
 Florida State University
 Florida, University of
 Flushing Hospital Medical Center
 Food and Drug Administration
 Fordham University College, Lincoln Center
 Franklin and Marshall College

George Washington University
 George Washington University Hospital
 Georgetown University
 Georgia, College of
 Georgia Tech
 Graduate Hospital

Hampshire College
 Harvard University
 Harvard Medical School
 Harvey Mudd College

Hawaii, University of
Houston, University of
Howard Hughes Medical Institute
Howard University
Hunter College

Illinois Institute of Technology
Illinois State University
Illinois, University of
Illinois, University of, Chicago College of
Medicine
Iowa State University
Iowa, University of

Johns Hopkins University
Johns Hopkins University School of
Medicine

Kaiser Permanente
Kansas, University of
Kansas, University of, Medical Center
Kentucky, University of
Kewalo Laboratory, Pacific Biomedical
Research Center

Lehigh University
Lehman College, CUNY
Lilly Research Labs
Los Alamos National Laboratory

Maryland, University of
Maryland, University of, School of Medicine
Massachusetts General Hospital
Massachusetts, University of, Amherst
Massachusetts, University of, Dartmouth
Massachusetts, University of, Medical School
Mayo Medical and Graduate Schools
Medical College of Georgia
Medical College of Pennsylvania
Meharry Medical College
Mercy Catholic Medical Center
Miami University
Miami, University of, School of Medicine
Michigan State University
Michigan, University of
Minnesota, University of
Missouri, University of, Columbia
Monell Chemical Senses Center
Montclair State University
Montefiore Medical Center
Mount Sinai Medical Center
Monterey Bay Aquarium Research Institute
Museum of Vertebrate Zoology

National Cancer Institute
National Institute of Environmental Health
Science
National Institutes of Health
National Institute of Neurological Disorders
and Stroke
New Jersey, University of Medicine and
Dentistry
New Mexico, University of
New York Medical College

New York State Institute of Basic Research in
Developmental Disabilities
New York, State University of, Buffalo
New York, State University of, Health
Science Center
New York, State University of, Stony Brook
New York University
New York University Medical Center
Noble Foundation
North Carolina State University
North Carolina, University of, Chapel Hill
North Texas, University of
North Texas, University of, Health Science
Center, Fort Worth
Northwestern University Medical School
Notre Dame, University of

Ohio, Medical College of
Old Shriners Hospital
Oregon Health Science University
Oregon, University of

Parmley Hearing Institute, Loyola University
of Chicago
Pennsylvania, University of
Pennsylvania, University of, School of
Medicine
Pittsburgh, University of
Pittsburgh, University of, School of Medicine
Point Loma Nazarene College
Pomona College
Princeton University
Procter & Gamble
Project HOPE
Puerto Rico, University of
Puerto Rico, University of, Medical Sciences
Purdue University

Queens College, CUNY

Rhode Island, University of
Rhône-Poulenc Rorer
Roche Institute of Molecular Biology
Roche Molecular Systems
Rochester, University of, Medical Center
Rochester, University of, School of Medicine
& Dentistry
Rockefeller University, The
Rush-Presbyterian—St. Luke's Medical
Center
Rutgers University

Scripps Research Institute
Smithsonian Tropical Research Institute
South Carolina, University of
South Florida, University of
Spelman College
St. Elizabeth's Hospital
St. Joseph Medical Center
Stanford University
Stockton State College
Swarthmore College
Syracuse University
Syracuse University Institute for Sensory
Research

Temple University
 Temple University Medical School
 Texas A&M University
 Texas, University of, Austin
 Texas, University of, Galveston
 Texas, University of, Houston
 Texas, University of, San Antonio
 Texas, University of, Medical Branch,
 Galveston
 Texas, University of, Southwestern Medical
 Center, Dallas
 Thomas Jefferson University
 TopoMetrix
 Tripler Army Medical Center

U.S. Department of State
 Union College
 Utah, University of

Vanderbilt University
 Virginia, University of
 Virginia, University of, School of Medicine

Wadsworth Center for Labs and Research
 Wake Forest University
 Washington Hospital Center
 Washington, University of
 Washington, University of, School of
 Medicine
 Wesleyan University
 West Florida, University of
 Whitehead Institute
 Whitney Laboratory
 Williams College
 Wisconsin, University of, Madison
 Wisconsin, University of, Medical School
 Wistar Institute
 Woods Hole Oceanographic Institution
 Worcester Foundation for Experimental
 Biology

Yale University
 Yale University School of Medicine

Foreign Institutions Represented

Aarhus, University of, Denmark
 AG Plant Biotech, India
 Alberta, University of, Canada
 Antwerp, University of, Belgium

Barcelona, Universitat de, Spain
 Basel, University of, Switzerland
 Basel, University of, Dental Institute,
 Switzerland
 Ben Gurion University, Beer Sheva, Israel
 Birmingham, University of, England
 Bologna University, Italy

Cairo University, Egypt
 Cambridge, University of, England
 Cape Town, University of, South Africa

Chile, University of
 CINVESTAV, Mexico
 CNRS, France
 Cologne, University of, Germany

Dalhousie University, Canada
 Dalhousie University Faculty of Medicine,
 Canada

EAWAG Kastanienbaum, Switzerland
 Ecole Polytechnique, France
 Edinburgh University, Scotland

Fredrich Miescher Institut, Switzerland

Glasgow, University of, Scotland

Hebrew University of Jerusalem, Israel
 Hospital for Sick Children, Canada

IBS-CNR, Italy
 INSERM, France
 Institute of Zoology, London, England
 Institut Pasteur de Tunis, Tunisia
 I.V.I.C., Venezuela
 I.I.B.C.E., Uruguay
 Instituto de Investigaciones Biologicas,
 Uruguay
 Instituto M. y M. Ferreyra, Argentina

Karolinska Institute, Sweden
 Kuwait University Faculty of Medicine,
 Kuwait
 Kyoto University, Japan

Laboratoire de Nutrition et Securite
 Alimentaire, France
 Lethbridge, University of, Canada
 Leuven, University of, Belgium
 Liverpool John Morris University, England
 Liverpool, University of, England

Madrid, Universidad Autonoma de, Spain
 Manchester, University of, England
 Montreal, Université de, Canada
 Marine Biological Association, England
 Max Planck Institute for Biophysics,
 Germany

Max Planck Institute for Brain Research,
 Germany
 Max Planck Institute Tübingen, Germany
 Max Planck Institute Göttingen, Germany
 Mainz University Medical Center, Germany
 McGill University, Canada
 Medical Research Council, England
 Melbourne, University of, Australia
 Montpellier, Université de, France
 Montreal General Hospital Research
 Institute, Canada

Nagoya University, Japan
 Naples, University of, Italy
 National Museums of Kenya, Kenya
 Newcastle, University of, England
 Novo Nordisk, Denmark

Oldenburg, University of, Germany
Osaka University, Japan
Oswaldo Cruz Institute, Brazil
Ottawa, University of, Canada
Oxford, University of, England

Pisa, University of, Italy

Queensland, University of, Australia
Queen's University, Ontario, Canada

Regensburg, University of, Germany
Rome, University of, Italy
Rouen, Université de, France

Saitama Cancer Center, Japan
Santiago, University of, Chile
Stockholm University, Sweden
Swiss Federal Institute, Switzerland

Tata Institute of Fundamental Research,
India
Technion, Faculty of Medicine, Israel
Tokyo Institute of Technology, Japan
Tokyo, University of, Japan
Tromsø, University of, Norway

UMIST, England
Universidad Nacional Autónoma de México,
Mexico
Universidad Simón Bolívar, Venezuela
University College London, England
Uppsala University, Sweden

Victoria, University of, Canada

Wageningen Agricultural University,
The Netherlands
Wales, University of, Cardiff, England
Warwick, University of, England
Waterloo, University of, Canada
Witwatersrand, South Africa



Year-Round Research Programs

Architectural Dynamics in Living Cells Program

Established in 1992, this program focuses on architectural dynamics in living cells—the timely and coordinated assembly and disassembly of macromolecular structures essential for the proper functioning, division, motility, and differentiation of cells; the spatial and temporal organization of these structures; and their physiological and genetic control. The program is also devoted to the development and application of powerful new imaging and manipulation devices that permit such studies directly in living cells and functional cell-free extracts. The Architectural Dynamics in Living Cells Program promotes interdisciplinary research and consists of resident core investigators and a cadre of adjunct members.

Resident Core Investigators

Inoué, Shinya, Distinguished Scientist
Oldenbourg, Rudolf, Associate Scientist
Stemmer, Andreas, Visiting Assistant Scientist

Staff

Knudson, Robert, Instrument Development Engineer
Leighton, Jane, Executive Assistant

Visiting Investigators

Arimoto, Rieko, Nikon Corporation, Tokyo, Japan
Fukui, Yoshio, Northwestern University Medical School
Inoué, Theodore D., Universal Imaging Corporation, West Chester, PA
Krendel, Mira, Rutgers University
Murray, John M., University of Pennsylvania
Roegiers, Fabrice, Station Zoologique, Villefranche-sur-Mer, France
Suzuki, Keisuke, Olympus Corporation, Hachioji, Japan
Terada, Horotoshi, Hamamatsu Photonics K.K., Hamamatsu City, Japan
Tran, Phong, University of North Carolina, Chapel Hill

Boston University Marine Program

Faculty

Atema, Jelle, Professor of Biology, Director
Dionne, Vincent, Professor of Biology, Associate Director for Graduate Studies
Humes, Arthur G., Professor of Biology Emeritus
Kaufman, Les, Associate Professor of Biology

Lobel, Phillip, Associate Professor of Biology
Tamm, Sidney L., Professor of Biology
Valiela, Ivan, Professor of Biology
Voigt, Rainer, Research Associate Professor

Staff

Corbett, Jean, Administrative Coordinator
Guillfoyle, Kerry, Course Coordinator
Hahn, Dorothy, Senior Administrative Secretary
Hall, Sheri, Program Manager
Pedersen, Jennifer, Program Assistant

Visiting Faculty and Investigators

Cleland, Thomas, University of California San Diego
D'Avanzo, Charlene, Hampshire College
Epstein, Slava, Northeastern University
Hinkle, Greg, MBL
Horridge, Adrian, Australia National Univ.
McFall-Ngai, Margaret, Univ. of Southern California
Mulsow, Sandor, Bedford Institute of Oceanography
Muscantine, Len, University of California Los Angeles
Read, Andrew, Woods Hole Oceanographic Institution
Rietsma, Carol, SUNY, New Paltz
Rommel, Sentiel, Smithsonian Institution
Rosenbaum, Joel, Yale University
Sardet, Christian, Villefranche Zoological Station
Simmons, William, Sandia National Laboratory
Solow, Andrew, Woods Hole Oceanographic Institution
Thayer, Charles, University of Pennsylvania
Wainwright, Norman, MBL
Ward, Nathalie, Center for Coastal Studies

Research Staff

Basil, Jennifer, Postdoctoral Investigator
Cohen, Anne, Postdoctoral Investigator
Delay, Rona, Postdoctoral Investigator
Eisthen, Heather, Postdoctoral Investigator
Foreman, Kenneth, Postdoctoral Investigator
Gerardo, Hortense, Postdoctoral Investigator
Gorka, Sancho, Research Assistant
Grasso, Frank, Postdoctoral Investigator
Lowrance, Courtney, Research Assistant
Nixon, Jennifer, Research Assistant
Soucy, Lori, Research Assistant
Seely, Brad, Visiting Research Assistant
Steele, Meg, Laboratory Assistant
Tomasky, Gabrielle, Research Assistant
Trott, Thomas, Postdoctoral Investigator

Graduate Students

PhD students

Balint, Claire
Batjakas, Ioannis
Behr, Peter
Dale, Jonathan
Economakis, Alistair
Farley, Lynda
Hauxwell, Jennifer
Herrold, Ruth
Lowe, Brian
Ma, Diana
McClelland, James
Oliver, Steven
Zhou, Qiao

MA students

Ashcraft, Susan
Bell, Kimberly
Bocking, Beatrice
Bugajski, David
Bugbee, Elizabeth
Cervino, James
DiNunno, Paul
Daniels, Heather
Decarie, Linette
Esquibel, Anthony
Heiskell, Mary Beth
Hopkins, Alyssa
Jenkins, Aaron
Keith, Lucy
Kerr, Lisa
Killen, Heather
Mason, Susan
Paganessi, Laura
Philibotte, Jason
Pinto-Torres, Sonia
Pitzer, Jocelyn
Pokoly, Melanie
Quinn, Catherine
Reilly, Christian
Roberts, Brian
Talariski, Jennifer
Timmer, Edward
Wittenberg, Kim
Wrights, Dionne

Summer 1995 Undergraduate Interns

Ahern, Jennifer
Anderson, Edward
Beech, Peter
Bermudes, David
Bittner, Kevin
Bohrer, Travis
Buckland-Nicks, John
Callaway, David
Chaplin, Sue Ann
Drake, Chaka
Eldridge, Robert
Evans, Tony
Gatso, Eguchi

Ithier, Warner
Linck, Richard
Lovell, Peter
Lyons, Julie
MacGregor, Catherine
Mann, David
Martinez, Nicole
Preisser, Matthew
Selkoe, Kim
Sheridan, Cecelia
Wintermyer, Margy
Wolfe, Cheryl
Wright, Amos

Undergraduate Students, Fall 1995

Abromaitis, Grace, Boston College
Alexander, David
Amico, Alyssa
Boyar, Marialena
Bula, Staci, Beloit
Cosman, Kristen
Diggin, Melissa
Dompé, Alvaro
Drucker, Samuel
Elliott, Shannon
Gallagher, Sheila
Gamble, Megan
Gettings, Jill, Beloit
Goldenberg, Hannah
Hard, Lorien
Hollmeyer, Gretchen
Hopkins, Alyssa
Howe, Aimee
Jarzowski, Joanne
Lanning, Eric
Lawler, Raymond
Lent, Emily
Lo, Francesca, Brown University
Martinez, Robert
Mole, Elizabeth
Panasik, Gina
Patel, Sonali
Peluso, Craig
Phelan, Patrick
Roberts, Jenny
Runde, Robert
Salomone, Anita, UMass, Amherst
Schrunk, Donald, Kenyon College
Shea, Keri
Tu, Sonja
Unger, David
Wakeheld, Joyce

Laboratory of Jelle Atema

Many organisms use chemical signals as their main source of information about the environment. These signals are transported in the marine environment by turbulent currents, viscous flow, and molecular diffusion. Receptor organs extract signals through various physical & biological filtering processes. Currently, the lobster with its exquisite sense of taste and smell, is our major model to study the signal filtering capabilities of the whole animal and the tuning properties of its receptor cells. Research focuses on food signals and

pheromones used in courtship and dominance, neurophysiology of receptor cells, behavior guided or modulated by chemical signals, computational models of odor plumes and neural filters, and underwater robotics.

Laboratory of Vincent Dionne

Odors are powerful stimuli. They can focus the attention, elicit behaviors (or misbehaviors), and even resurrect forgotten memories. These actions are directed by the central nervous system, but they depend upon the initial transduction of chemical signals by olfactory receptor neurons in the nasal passages. More than just a single process appears to underlie odor transduction, and the intracellular pathways that are used are far more diverse than once thought. Hundreds of putative odor receptor molecules have been identified that work through several different second messengers to modulate the activity of various types of membrane ion channels. Our studies are being conducted with aquatic salamanders using amino acids and other soluble chemical stimuli which these animals perceive as odors. Using electrophysiological and molecular approaches, the research examines how these cellular components produce odor detection, and how odors are identified and discriminated.

Laboratory of Arthur G. Humes

Research interests include systematics, development, host specificity, and geographical distribution of copepods associated with marine invertebrates. Current research is on taxonomic studies of copepods from invertebrates in the tropical Indo-Pacific area, and poecilostomatoid and siphonostomatoid copepods from deep-sea hydrothermal vents and cold seeps.

Laboratory of Phillip Lobel

Fishes are the most diverse vertebrate group and provide opportunities to study many aspects of behavior, ecology, and evolution. We primarily study how fish are adapted to different habitats and the behavioral ecology of species interactions. Current research focuses on fish acoustic communication. We are also conducting a long-term study of the marine biology of Johnston Atoll, Central Pacific Ocean. Johnston Atoll has been occupied continuously by the military since the 1930s and provides a unique opportunity for assessing the biological impacts of pollution. Unlike a city harbor, chemical spills at the atoll are documented as to location, date, and amount.

Laboratory of Sidney Tamm

Marine model systems offer unique experimental advantages for solving basic problems in cell biology and physiology. In particular, comb jellies (ctenophores), important members of the marine zooplankton, possess the largest cilia and smooth muscles in nature, a simple nervous system, and interesting feeding and locomotory behaviors. We use ctenophores to investigate the mechanism of ciliary movement and ciliary coordination, the neural and ionic control of cilia (particularly stimulus-evoked intraciliary calcium transients and distribution of ciliary calcium channels), geotaxis and mechanosensory transduction by motile cilia statocyst, structure and function of smooth muscle, double-modality sensory receptors and the cytoskeleton, and evolution of neurotransmitters, and a new type of reversible cell-cell adhesion that closes the mouth of *Beroë*, a voracious predator of other ctenophores. In addition, we use a termite protozoan with a continuously rotating head to investigate novel types of cell motility, the fluid nature of cell membranes, and remarkable prokaryotic-eukaryotic motility symbioses.

Laboratory of Ivan Valiela

Our major research activity involves the Waquoit Bay Land Margin Ecosystems Research Project. This work examines how human activity in coastal watersheds (including landscape use and urbanization) increases nutrient loading to groundwater and streams. Nutrients in groundwater are transported to the sea, and, after biogeochemical transformation, enter coastal waters. There, increased nutrients bring about a series of changes. The Waquoit Bay LMER is designed to help understand and model the coupling of land use and consequences to receiving waters, to study the processes involved, and to assess consequences and opportunities for coastal management.

A second long-term research topic is the structure and function of salt marsh ecosystems, including the processes of predation, herbivory, decomposition, and nutrient cycles.

Calcium Imaging Laboratory

This laboratory investigates the roles of calcium patterns in development. Our main tool uses the aequorins, a family of luminescent proteins ultimately obtained from a jellyfish and long studied by Dr. Osamu Shimomura at the MBL. Aequorins can either be microinjected into cells or transgenically expressed without disturbing function or development. The patterns of luminescence that are emitted by aequorinated cells reveals changing patterns and levels of free calcium within the cell or its progeny. Much of what we know about the roles of calcium in development has been obtained with the aequorins.

The four systems under present or planned investigation are the *Drosophila* egg (in collaboration with Drs. Lynn Cooley and Carl Hashimoto at Yale), the zebrafish egg (in conjunction with Dr. Roger Hanlon at the MBL), the fucoid egg (in collaboration with Dr. Ken Robinson at Purdue) and the cellular slime mold, *Dictyostelium*.

Staff

Steele, Margaret, Research Assistant
Créton, Robert, Research Associate
Jaffe, Lionel, Senior Scientist

Center for Advanced Studies in the Space Life Sciences at the MBL

(supported by the National Aeronautics and Space Administration)

The Marine Biological Laboratory and NASA have formed a partnership with the establishment of a NASA-sponsored Center for Advanced Studies in the Space Life Sciences at the MBL. This center serves as an interface between NASA and the basic biological science community, addressing issues of mutual interest. A series of symposia, workshops, and seminars will be held at the MBL to advise NASA on a wide variety of topics in the life sciences. Special attention will be directed at examining how gravity and its control impact biological processes, and how variations in gravity can be used as a probe to better understand such processes. This setting will provide a forum for scientists to think and to discuss, often for the first time, the role that gravity may play in fundamental cellular and physiologic processes. Meetings at the MBL will inform the community of research opportunities in the life sciences that are of interest to NASA. In addition to meetings, a newsletter will be published to disseminate this information to a wider audience.

Staff

Crosby, Ann, Administrative Assistant
Dawidowicz, Lenny, Administrator



The Ecosystems Center

The Center carries out research and education in ecosystems ecology. Terrestrial and aquatic scientists work in a wide variety of ecosystems ranging from the streams, lakes, and tundra of the Alaskan Arctic (limits on plant primary production) to sediments of Massachusetts Bay (controls of nitrogen cycling), to forests in New England (effects of soil warming on carbon and nitrogen cycling) and South America (effects on greenhouse gas fluxes of conversion of rain forest to pasture), and to large estuaries in the Gulf of Maine (effects on the plankton and benthos of nutrients and organic matter in stream runoff). Many projects, such as those dealing with carbon and nitrogen cycling in forests, streams, and estuaries, use the stable isotopes ^{13}C and ^{15}N to investigate natural processes. A mass spectrometer facility is available. Data from field and laboratory research are used to construct mathematical models of ecosystem processes. Some of these models are combined with geographically referenced data to produce estimates of how environmental changes affect key ecosystem indexes such as net primary productivity and carbon storage throughout the world's terrestrial biosphere. The results of the Center's research are applied, wherever possible, to the questions of the successful management of the natural resources of the Earth. In addition, the ecological expertise of the staff is made available to public affairs groups and government agencies who deal

with problems such as acid rain, coastal eutrophication, and possible carbon dioxide-caused climate change. There are opportunities for postdoctoral fellows and graduate students.

Administrative Staff

Hobbie, John E., Co-Director
 Melillo, Jerry M., Co-Director
 Berthel, Dorothy J., Administrative Assistant
 Donovan, Suzanne J., Executive Assistant
 Monahan, A. Jean, Administrative Assistant
 Nunez, Guillermo, Research Administrator
 Pennington, Susan M., Administrative Assistant
 Seifert, Mary Ann, Administrative Assistant
 Scanlon, Deborah G., Executive Assistant, LMER Coordinator

Scientific Staff

Hobbie, John E., Senior Scientist
 Melillo, Jerry M., Senior Scientist
 Peterson, Bruce J., Senior Scientist
 Shaver, Gaius R., Senior Scientist
 Giblin, Anne E., Associate Scientist
 Hopkinson, Charles S., Associate Scientist
 Nadelhoffer, Knute J., Associate Scientist
 Deegan, Linda A., Associate Scientist
 Rastetter, Edward B., Associate Scientist
 Steudler, Paul A., Senior Research Specialist
 McGuire, A. David, Research Associate
 McKane, Robert B., Research Associate
 Neill, Christopher, Research Associate
 Foreman, Kenneth H., Associate Director of Environmental Studies Program

Educational Staff Appointments

Bret-Harte, Marion S., Postdoctoral Research Associate
 Currie, William, Visiting Postdoctoral Scholar, U.S. Department of Agriculture
 Fernandes, David N., Postdoctoral Research Associate
 Holmes, Robert M., Postdoctoral Research Associate
 Johnson, Loretta C., Postdoctoral Research Associate
 Pan, Yude, Postdoctoral Research Associate
 Suehlitz, Marc, NOAA Global Climate Change Postdoctoral Fellow
 Tian, Hanqin, Postdoctoral Research Associate
 Weaver, Melissa J., Postdoctoral Research Associate
 Williams, Mathew, Postdoctoral Research Associate
 Vallino, Joseph J., Postdoctoral Research Associate
 Xiao, Xiangming, Postdoctoral Research Associate

Technical Staff

Bahr, Michele P., Research Assistant
 Bettez, Neil D., Research Assistant
 Buffam, Ishi D., Research Assistant
 Canary, Jana D., Research Assistant
 Catricala, Christina E., Research Assistant
 Dornblaser, Mark M., Research Assistant
 Downs, Martha R., Research Assistant
 Garritt, Robert H., Senior Research Assistant
 Harvey, Christopher J., Research Assistant
 Helfrich III, John V. K., Senior Research Assistant
 Kicklighter, David W., Senior Research Assistant
 Kwiatkowski, Bonnie L., Research Assistant
 Laundre, James A., Senior Research Assistant

Micks, Patricia, Research Assistant
Newkirk, Kathleen M., Research Assistant
Nolin, Amy L., Research Assistant
Regan, Kathleen M., Research Assistant
Ricca, Andrea, Research Assistant
Schwamb, Carol, Laboratory Assistant
Tholke, Kristin S., Research Assistant
Tucker, Jane, Senior Research Assistant
Wollheim, Wilfred M., Research Assistant

Consultants

Bowles, Francis P., Research Systems Consultant, Research Designs
Bowles, Margaret C., Administrative Consultant
Fry, Brian D., Visiting Consultant, Florida International University

Visiting Scientists and Scholars

Galloway, James, Visiting Scientist, University of Virginia
Gundersen, Per, Visiting Scientist, Danish Forest and Landscape
Research Institute, Ministry of Energy and Environment
Kaduk, Jrg, Visiting Scientist, Max Planck Institute for Meteorology,
Hamburg, Germany
LeCat, Camille, Visiting Student, Universit de Technologie de
Compiene
Piccolo, Marisa, Visiting Scientist, CENA, University of So Paulo
Sauf, Walter, Visiting Scientist, Max Planck Institute for Meteorology,
Hamburg, Germany
Schmidt, Inger Kappel, Visiting Scientist, University of Copenhagen
Sitch, Stephen, Visiting Postdoctoral Candidate, University of Lund
Zago, Christina, Visiting Scholar, Instituto per lo Studio della
Dinamica delle Grandi Masse, ISDGM, Venice, Italy

Laboratory of Aquatic Animal Medicine and Pathology

The laboratory provides diagnostic, consultative research, and educational services to the institutions and scientists of the Woods Hole community concerned with marine animal health. Diseases of wild, captive, and cultured animals are investigated.



Staff

Abt, Donald A., Director and The Robert R. Marshak Term Professor
of Aquatic Animal Medicine and Pathology, School of Veterinary
Medicine, University of Pennsylvania
Bullis, Robert A., Research Assistant Professor of Microbiology,
University of Pennsylvania
Leibovitz, Louis, Director Emeritus
McCaflerty, Michelle, Histology Technician, University of
Pennsylvania
Moniz, Priscilla C., Administrative Assistant
Smolowitz, Roxanna M., Senior Research Investigator in Pathology,
University of Pennsylvania
Wadman, Elizabeth A., Microbiology Technician, University of
Pennsylvania

Laboratory of Aquatic Biomedicine

This laboratory investigates tumors of marine invertebrates by focusing on leukemias of soft shell clams. Monoclonal antibodies developed by this laboratory are being used to diagnose clam leukemia, identify and characterize a tumor-specific protein, and differentiate other leukemias in bivalve molluscs. The availability of molecular probes has enabled the laboratory to examine the expression of the p53 gene in both normal and leukemia cells. The impact of pollutants on clam leukemogenesis is currently being studied with an emphasis on regional superfund sites.

Staff

Reinisch, Carol L., Associate Scientist, MBL, and Chairperson,
Department of Comparative Medicine, Tufts University School of
Veterinary Medicine
Barker, Colin, Research Assistant

Visiting Scientists

Brand, Dawna, Department of Environmental Studies, University of
Victoria, British Columbia
Strandberg, John, Department of Comparative Medicine, The Johns
Hopkins School of Medicine
Powell, DeLois, Department of Biology, University of Maryland,
Eastern Shore

Students

Smith, Cynthia, Tufts University School of Veterinary Medicine
Harper, Claudia, Tufts University School of Veterinary Medicine

Laboratory of Cell Biochemistry

This laboratory uses cell and molecular biological methods to study the regulation of gene expression in marine fish. Current emphasis is on gene products involved in heme metabolism including: (a) aminolevulinic synthase, the first and rate determining enzyme of heme production; (b) cytochrome P450, a heme-requiring catalyst for oxidation of hydrophobic chemicals; and (c) heme oxygenase, a stress-induced, microsomal enzyme that catalyzes the first reaction of heme degradation. The expression of all three enzymes is affected by endogenous and pharmacological agents as well as xenobiotics and carcinogens. We have cloned and sequenced cDNAs for both the erythroid and housekeeping forms of aminolevulinic synthase, have developed specific probes for cytochrome P450, and, by RT-PCR, are generating a homologous probe for heme oxygenase. When that is completed, we will have a battery of fish-specific molecular biological

reagents that can be used to monitor environmental effects on heme biosynthesis, utilization, and degradation. It is expected that such simultaneous analyses will be much more informative than measurements made on only one aspect. We recently have shown that isolated fish hepatocytes regulate heme biosynthesis in a manner resembling that in terrestrial vertebrates (including humans), and we are using primary cultures of fish hepatocytes to address some long-standing biomedical questions regarding the mechanisms of that regulation. Because preliminary sequence alignments indicate that aminolevulinate synthase has an interesting evolutionary history that bears on the endosymbiont hypothesis for the origin of animal mitochondria, these studies will be extended to include comparative molecular biology of aminolevulinate synthases from invertebrates and lower eukaryotes.

Staff

Cornell, Neal W., Senior Scientist
Faggart, Maura A., Research Assistant
Martin, Holly A., Research Assistant
Macarro, Jackie, Laboratory Assistant

Visiting Scientist

Fox, T. O., Harvard Medical School

Laboratory of Cell Communication

Established in 1994, this laboratory is devoted to the study of intercellular communication. The research focuses on the cell-to-cell channel, a membrane channel built into the junctions between cells. This channel provides one of the most basic forms of intercellular communication in organs and tissues. The work is aimed at the molecular physiology of this channel, in particular, at the mechanisms that regulate the communication. Electrophysiological-, fluorescent-tracer-, and molecular biological techniques are used to this end. As was recently discovered in this laboratory, the channel is the conduit of growth-regulating signals. It is instrumental in a basic feedback loop whereby cells in organs and tissues control their number; in a variety of cancer forms it is crippled. Work is aimed now at the mechanisms of growth control and at correcting cancer growth by transferring the gene for the cell-to-cell channel protein from normal cells into the cancer cells. Molecular genetic techniques are used in this endeavor.

Staff

Werner Loewenstein, Senior Scientist
Birgit Rose, Senior Scientist
Tracy Jillson, Research Assistant

Laboratory of Barbara and Bruce Furie

γ -Carboxyglutamic acid is a calcium-binding amino acid well-distributed among mammalian species, but the search for this amino acid in invertebrates has not been revealing, with a single exception: toxins from the predatory marine cone snails, *Conus*. The biosynthesis of γ -carboxyglutamic acid in the mammalian vitamin K-dependent proteins has been studied extensively in our laboratory at Tufts-New England Medical Center, with recent emphasis on the cloning, structure, and enzymology of the γ -carboxylase and the mechanistic role of vitamin K. The synthesis of this amino acid is critical for normal blood coagulation in that all of the vitamin K-dependent blood coagulation proteins contain γ -carboxyglutamic acid, an amino acid that is required for biological activity.

With the discovery of γ -carboxyglutamic acid in conotoxins isolated from the toxins of the venoms of predatory marine snails belonging to the genus *Conus*, the question arises as to the pathway of biosynthesis of this amino acid, the potential role of vitamin K in its synthesis and comparison of invertebrate and mammalian γ -carboxylation systems. Although our primary laboratory in Boston has been engaged in the study of the mammalian blood coagulation proteins that contain γ -carboxyglutamic acid, and the biosynthesis of these proteins, the new MBL laboratory is an initiative to study invertebrate γ -carboxyglutamic acid biosynthesis in a unique marine animal that synthesizes γ -carboxyglutamic acid. The laboratory is developing an *in vitro* assay for γ -carboxyglutamic acid synthesis by the endogenous *Conus* carboxylase using synthetic peptides based upon the structure of conantokin-G and conantokin-T. With this assay, a purification scheme for the carboxylase from *Conus* tissue will be developed. The long-term goals are to clone this enzyme, to determine the amino acid sequence homology to the mammalian carboxylase, and to understand its mechanism of action in fixing carbon dioxide to glutamic acid.

Staff

Barbara Furie, Visiting Scientist
Bruce Furie, Visiting Scientist

Laboratory of Roger Hanlon

This Laboratory investigates the behavior of cephalopods. Studies of observational learning capabilities are currently being conducted, as are studies on reproductive strategies that include agonistic behavior, female mate choice, and sperm competition. The latter studies involve DNA fingerprinting to determine paternity and help assess alternative mating tactics. Complementary field studies are conducted locally and on coral reefs. The functional morphology and neurobiology of the chromatophore system of cephalopods are also studied on a variety of cephalopod species, and image analysis techniques are being developed to study crypsis and the mechanisms that enable cryptic body patterns to be neurally regulated by visual input.

Staff

Hanlon, Roger, Senior Scientist
Ashcraft, Susan, Research Assistant

Visiting Investigators

Claes, Graduate Student, Northeastern University
Jenkins, Aaron, Student, Boston University Marine Program
Wittenberg, Kim, Graduate Student, Boston University Marine Program

Laboratory of Shinya Inoué

Study of the molecular mechanism and control of mitosis, cell division, cell motility, and cell morphogenesis, with emphasis on biophysical studies made directly on single living cells, especially developing eggs in marine invertebrates. Development of biophysical instrumentation and methodology, such as polarization optical and video microscopy and digital image processing techniques, and exploration of their underlying theory are an integral part of the laboratory's effort.

Staff

Inoué, Shinya, Distinguished Scientist
 Knudson, Robert, Instrument Development Engineer
 Leighton, Jane, Executive Assistant
 Maccaro, Jackie, Laboratory Assistant
 Stemmer, Andreas, Visiting Assistant Scientist

Laboratory of Alan M. Kuzirian

Our research explores the functional morphology and ultrastructure of various organ systems in opisthobranch molluscs. The program includes mariculture of nudibranch, *Hermisenda crassicornis*, with emphasis on developing reliable culture methods for rearing and maintaining the animal as a research resource. The process of metamorphic induction by natural and artificial inducers is also being explored in an effort to understand the processes involved and as a means to increase the yield of cultured animals. Morphologic studies stress the ontogeny of neural and sensory structures associated with the photic and vestibular systems that have been used in learning and memory studies. Also being investigated are the spatial and temporal occurrence of regulatory and transmitter neurochemicals in the central and peripheral nervous system.

Concurrent with these morphologic studies is the development of a new technique to obtain and reconstruct serial block face images (SBFI) of epoxy-embedded or cryoprepared tissues sectioned or freeze-fractured or freeze-etched inside an SEM by an *in situ* miniature ultramicrotome. Also, collaborative efforts are being conducted toward developing and testing an inexpensive and durable silicon microtomy knife.

Collaborative research includes histochemical investigations on strontium's role in initiating calcification in molluscan embryos (shell and statoliths), as well as immunocytochemical labeling of cell-surface and secretory product antigens using mono- and polyclonal antibodies on *Hermisenda* sensory and neurosecretory neurons *in situ*, and in cell culture. Toxicity studies on lead effects on *Hermisenda* learning and feeding, and physiology of cultured neurons are also being conducted.

Additional collaborative research includes DNA fingerprinting using RAPD-PCR techniques in preparation for isogenic strain development of laboratory-reared *Hermisenda* and hatchery-produced bay scallops (*Argopecten irradians*) with distinct phenotypic markers for rapid field identification. The functional morphology and physiology of digestion in various marine metazoan invertebrates are being described. Systematic and taxonomic studies of nudibranch molluscs are also of interest.

Staff

Kuzirian, Alan M., Associate Scientist

Visiting Investigators

Bumann, Dirk
 Hemant Chikarmane, Research Scientist, Aphios, Assistant Scientist, MBL
 Leighton, Stephen B., Biomedical Engineering & Instrumentation Branch, NCRR-NIH

Laboratory of Norman Wainwright

The mission of the laboratory is to understand the molecular defense mechanisms exhibited by marine invertebrates in response to invasion by bacteria, fungi, and viruses. The primitive immune systems demonstrate unique and powerful strategies for survival in

diverse marine environments. The key model has been the horseshoe crab *Limulus polyphemus*. *Limulus* hemocytes exhibit a very sensitive LPS-triggered protease cascade which results in blood coagulation. Several proteins found in the hemocyte and hemolymph display microbial binding proteins that contribute to antimicrobial defense. Commensal or symbiotic microorganisms may also augment the antimicrobial mechanisms of macroscopic marine species. Secondary metabolites are being isolated from diverse marine microbial strains in an attempt to understand their role. Microbial participation in oxidation of the toxic gas hydrogen sulfide is also being studied.

Staff

Wainwright, Norman, Senior Scientist
 Barry, Kevin, Research Assistant
 Child, Alice, Research Assistant
 Kreiling, Jill, Postdoctoral Research Associate

Visiting Investigators

Anderson, Porter, University of Rochester
 Levin, Jack, University of California

Laboratory of Rudolf Oldenbourg

The laboratory develops advanced instrumentation in light microscopy and investigates physical optics relevant to microscope imaging for high resolution studies of architectural dynamics in living cells and cell components. The current focus of the laboratory is the development of a new polarized light microscope that combines microscope optics with electro-optical components, video, and digital image processing for fast analysis of specimen birefringence over the entire viewing field at the highest resolution of the light microscope. Biological systems currently investigated with the new pol-scope are fertilized eggs of the sea urchin, newt lung epithelial cells, microtubule *in vitro* preparations and lyotropic liquid crystals.

Staff

Oldenbourg, Rudolf, Associate Scientist
 Maccaro, Jackie, Laboratory Assistant
 Knudson, Robert, Instrument Development Engineer

Laboratory of Sensory Physiology

Members of this laboratory have conducted research on various facets of vision since 1973. Current investigations focus on vertebrate retinal photoreceptors. Light microscopy is used to study physical optical properties, such as birefringence, dichroism, and visual pigment absorbance. The chemical basis of color vision is investigated principally with UV/VIS absorption spectroscopy. One aim is a thorough understanding of the chemistry that underlies spectral tuning. Other objectives are related to biophysical mechanisms that permit polarized light detection and wavelength-dependent discrimination in the ultraviolet and visible spectra.

Staff

Harosi, Ferenc I., Senior Scientist, MBL, and Boston University School of Medicine

Laboratory of Osamu Shimomura

Biochemical mechanisms involved in the bioluminescence of various luminous organisms are investigated. Based on the results

obtained in this laboratory, improved forms of bioluminescent probes are designed and produced for the measurements of intracellular free calcium and superoxide anion.

Staff

Shimomura, Osamu, Senior Scientist, MBL, and Boston University School of Medicine
Shimomura, Akemi, Research Assistant

Laboratory of Robert B. Silver

This laboratory studies how living cells make decisions. The focus of the research, typically using marine models, is on two main areas: the role of calcium in the regulation of mitotic cell division (sea urchins, sand dollars, etc.) and structure/function relationships of hair cell stereociliary movements in vestibular physiology (oyster, toadfish). Other related areas of study, *i.e.*, synaptic transmission (squid), are also pursued. Tools include video light microscopy, multispectral and very high speed (sub-millisecond frame rate) photon counting video light microscopy, telemanipulation of living cells and tissues, and modeling of decision processes. A cornerstone of the laboratory's analytical efforts is high performance computational processing and analysis of video light microscopy images. With luminescent, fluorescent, and absorptive probes both empirical observation and computational modeling of cellular, biochemical and biophysical processes permit interpretation and mapping of space-time patterns of intracellular chemical reactions and calcium signalling in living cells. A variety of *in vitro* biochemical, biophysical, and immunological methods are used. In addition to fundamental biological studies, the staff designs and fabricates optical hardware, and designs software for large video image data processing, analysis, and modeling.

Staff

Silver, Robert, Associate Scientist
Crossin, Glenn, Laboratory Assistant
Fripp, William, Research Assistant
Hopkins, James, Research Assistant
Knudson, Robert, Instrument Engineer
Maccaro, Jackie, Laboratory Assistant

Interns

Brown, Angela, REU Intern, Georgia Institute of Technology
Speck, Alexandra, REU Intern, Columbia University

Visiting Scientists

Att, Maxim, MikroPhotonische Universitat von CZI
Kelley, Brian, Graduate Research Assistant, Cornell University
Palevitz, Lara, Cornell University
Reeves, Anthony, Cornell University
Stromboli, Emilio, Stazione de Napoli

Laboratory of Raquel Sussman

We investigate the molecular mechanism of DNA damage-inducible functions in *E. coli*. Present studies deal with novel genes that affect radiation-induced mutagenesis and analysis of RecA functions. In addition, we have been developing techniques for genomic mapping and collaborating in the isolation of neuronal genes in squid.

Staff

Sussman, Raquel, Associate Scientist

Visiting Investigator

Berberian, Graciela, Instituto de Investigacion Medica, Cordoba, Argentina

The Marine Resources Center

The Marine Resources Center is one of the world's most advanced facilities for maintaining and culturing aquatic organisms essential to advanced biological, biomedical and ecological research. Service and education also play important and complementary roles in this 32,000-square-foot, state-of-the-art facility.

The MRC and its life support systems have already increased the ability of MBL scientists to conduct research and have inspired new concepts in scientific experiments. Vigorous research programs focusing on basic biological and biomedical aquatic models are currently being developed at the Center. These programs will enhance and build upon the MRC's existing research activities by the University of Pennsylvania's Laboratory of Aquatic Animal Medicine and Pathology (LAAMP) and in the Laboratory of Roger Hanlon.

In addition to research, the MRC provides a variety of services to the MBL community through its Aquatic Resources Division (formerly the Marine Resources Department), the Water Quality and System Engineering Division, the Administrative Division, and the Laboratory for Marine Animal Health.

Educational and research opportunities are available at the facility to postdoctoral fellows, to graduate and undergraduates, and to established investigators. Students and investigators will find that the MRC's unique life support and seawater engineering systems make this a favorable environment in which to conduct masters and doctoral theses and independent research using a variety of aquatic organisms and flexible tank space for customized experimentation on live animals. Prospective students and investigators should contact the Director of the MRC for further information.

The MRC also hosts several courses annually: the AQUAVEIS® courses sponsored by LAAMP, and an aquaculture course, the theme of which changes yearly according to regional and national interests.



Staff

Hanlon, Roger, Director
Ashcraft, Susan, Research Assistant
Kuzirian, Alan, Acting Director

Visiting Investigators

Claes, Graduate Student, Northeastern University
Jenkins, Aaron, Student, Boston University Marine Program
Spotte, Stephen, University of Connecticut
Wittenberg, Kim, Graduate Student, Boston University Marine Program

Molecular Evolution of Genomes

The genome of the bacterium *Escherichia coli* contains all the information required for a free-living chemoautotrophic organism to live, adapt, and multiply. The information content of the genome can be dissected from the point of view of understanding the role of each gene and gene product in achieving these ends. The many functions of *E. coli* have been organized in a hierarchical system representing the complex physiology and structure of the cell. In collaboration with Dr. Peter Karp of SRI International, an electronic encyclopedia of information is being constructed on the genes, enzymes, metabolism, transport processes, regulation, and cell structure of *E. coli*. The interactive EcoCyc program is now publically available and currently has graphical hypertext displays, including literature citations, on nearly all of *E. coli* metabolism, all genes and their locations, a hierarchical system of cell functions and some regulation processes. This work is continuing.

In addition, the *E. coli* genome contains valuable information on molecular evolution. We are analyzing the sequences of proteins of *E. coli* in terms of their evolutionary origins. By grouping like sequences and tracing back to their common ancestors, one learns not only about the paths of evolution for all contemporary *E. coli* proteins, but one extends even further back before *E. coli*, traversing millenia to the earliest evolutionary times when a relatively few ancestral proteins served as ancestors to all contemporary proteins of all living organisms, not simply *E. coli*. The nearly complete genome sequence of *E. coli* and sophisticated sequence analysis programs permit us to identify evolutionary related protein families, determining ultimately what kinds of unique ancestral sequences generated all of present-day proteins.

Staff

Riley, Monica, Senior Scientist
Crossin, Glenn, Research Assistant I
Pelligriini-Toole, Alida, Research Assistant II

National Vibrating Probe Facility

The twofold purpose of the National Vibrating Probe Facility is to develop and make available techniques for the non-invasive measurement of transmembrane ion flux. Since the early 1980s, when this NIH-Biotechnology Resource Center was established at the MBL, a number of probes have been introduced: the vibrating potential difference probe (NVP_{pd}), a non-invasive wire voltage probe; the ion-selective probe (NVP_i); the oxygen probe (NVP_o); and the BioKelvin aerial probe (NVP_{bk}).

Vibrating a single, and therefore self-referencing, electrode has revolutionized the study of electric fields associated with living tissue. The NVP_{pd} is the most sensitive, measuring nanovolt fields relating to net current flow across membranes of tissues and cells. The NVP_i, one

of the few methods available for measuring the movement of ions involved in non-electrogenic transport, for example the activity of some pumps and porters, has the added advantage of measuring net flux of individual ions and, being based on commercially available ionophores, is broadly applicable. Although originally developed to study transmembrane calcium flux, to date ionophores for potassium and hydrogen have been successfully used with development studies completed for magnesium and chloride.

Highlights of recent work include the use of the *vas deferens* of rat to study the acidification of the male reproductive tract by a proton-pumping ATPase; studies of the physiological functions of cation currents using isolated *Aplysia* bag cell neurons; measurements of ion fluxes in mouse pre-implantation embryos; and a report on acetylcholine induced calcium fluxes across the sarcolemma of an echinoderm smooth muscle.

During this year, first recordings were made with the two newer systems, the oxygen probe and the BioKelvin aerial probe. The NVP_o, developed for the study of cell respiration, was used to make the first measurements of oxygen uptake by a single neuron; the NVP_{bk}, designed for measurement of weak fields around tissue in a gaseous environment, has been used to successfully measure electric fields around corn coleoptiles showing both photo and geotactic responses.

Staff

Jaffe, Lionel F., Director Emeritus
Smith, Peter J. S., Director
Hammar, Katherine, Research Assistant
Land, Stephen C., Postdoctoral Research Associate
McLaughlin, Jane A., Research Assistant
Sanger, Richard H., Senior Electronics Technician

Visiting Investigators

Baikie, Iain D., Robert Gordon University, Scotland
Breton, Sylvie, Harvard Medical School/Massachusetts General Hospital
Brown, Dennis, Harvard Medical School/Massachusetts General Hospital
Collier, Kimberley A., McMaster University, Canada
Demarest, Jeffery R., Juniata College
Devlin, C. Leah, Penn State
Eikenberry, Stephen J., Juniata College
Faszewski, Ellen, University of Massachusetts, Amherst
Gebhardt, Kelley A., Juniata College
Hill, Susan D., Michigan State
Holbrook, Pamela G., Universite de Montpellier II, France
Howes, Elizabeth, A., The Babraham Institute, England
Keefe, David, Yale University School of Medicine
Kunkel, Joseph G., University of Massachusetts, Amherst
Malchow, R. Paul, University of Illinois
Misiaszek, Christine F., McMaster University, Canada
Morgan, James L. M., University of Arkansas
Pepperell, John, Yale University Medical School
Tytell, Michael, Wake Forest University

Program in Comparative Molecular Biology and Evolution

The Program in Comparative Molecular Biology and Evolution employs comparative phylogenetic studies of genes and genomes to define patterns of evolution that gave rise to contemporary biodiversity on the planet earth. We are especially interested in discerning how the eukaryotic cell was invented as well as the identity

of microbial groups that were ancestral to animals, plants, and fungi. We take advantage of the extraordinary conservation of ribosomal RNAs to define phylogenetic relationships that span the largest of evolutionary distances. These studies have overhauled traditional eukaryotic microbial classification systems. We have discovered new evolutionary assemblages that are as genetically diverse and complex as plants, fungi, and animals. The nearly simultaneous separation of these eukaryotic groups (described as the eukaryotic "Crown") occurred approximately one billion years ago and was preceded by a succession of earlier diverging protist lineages, some as ancient as the separation of the prokaryotic domains. At the same time this database provides a powerful tool for the newly emerging discipline of molecular ecology. Using the ribosomal RNA database and nucleic acid based probe technology, it is possible to detect and monitor microorganisms including those that cannot be cultivated in the laboratory. This strategy has revealed new habitats and major revelations about geographical distribution of microorganisms.

More recently we have initiated a program to sample genomic diversity from eukaryotic microorganisms that do not have mitochondria. We previously demonstrated that these taxa represent

some of the earliest diverging lineages in the evolutionary history of eukaryotes. The objective is to develop a set of additional molecular markers for studying molecular evolution. These will be invaluable in unraveling sudden evolutionary radiations that cannot be resolved by rRNA comparisons and will provide insights into the presence or absence of important biochemical properties in the earliest ancestors common to all eukaryotic species.

Staff

Sogin, Mitchell L., Director and Senior Scientist
Hinkle, Greg, Postdoctoral Research Associate
Morrison, Hilary G., Postdoctoral Research Associate
Silberman, Jeffrey, Postdoctoral Research Associate

Visiting Investigators

Bahr, Michele, Ecosystems Center
Barnhisel, Rac, Postdoctoral Sloan Fellow
Collins, Allen, University of California, Berkeley



Honors

Friday Evening Lectures

June 16	Robert J. Full, University of California, Berkeley "Diversity Enables Discovery: Lessons From Many-Legged Locomotors as Inspiration for Robot Design"
June 23	Baldomero M. Olivera, The University of Utah "Using Venoms of the Deadly Cone Snails to Study the Brain"
June 30	Sharon R. Long, Howard Hughes Medical Institute and Stanford University "Life in the Underground: How Bacteria and Plants Communicate by Signal Exchange" (Monsanto Lecture)
July 7	William E. Paul, National Institutes of Health "Confronting AIDS: A New Paradigm for Responding to Major Diseases" (Glassman Lecture)
July 14	Robert D. Goldman, Northwestern University "Cellular Architecture: Form and Function in Health and Disease"
July 20, 21	John G. Nicholls, University of Basel "Repair and Regeneration of the Central Nervous System After Injury:" "Cellular and Molecular Aspects of Regeneration" and "Growth of Nerve Fibers Across Lesion of Neonatal Mammalian Spinal Cord in Culture" (Forbes Lectures)
July 28	Eve Marder, Brandeis University "Dynamic Modulation of Neurons and Networks" (Lang Lecture)
August 4	Susan S. Taylor, University of California, San Diego "Protein Kinases: Critical Switches in Signal Transduction"
August 11	Andrew H. Knoll, Harvard University "The Context of Early Animal Evolution: Time, Genealogy, and Environment"

Fellowships and Scholarships

In 1995, the MBL awarded research fellowships to 23 scientists from around the world. These fellows' research topics ranged from studying immune system precursors in horseshoe crabs to understanding cognitive function with the aid of computer modeling to examining cardiac muscle contractions in molluscs after being treated with calcium channel blocker drugs used to control hypertension in humans. The MBL also awarded scholarships to 146 students in the MBL's summer courses.

The Science Writing Fellowships Program, now in its 10th year, brings print and broadcast journalists to the MBL each summer to participate in a hands-on laboratory course in cell and molecular biology followed by assignments in the courses and with independent investigators. Fellowships were awarded to 13 journalists in 1995.

In 1995, donors provided \$158,760 in support of the research fellowship program, \$107,000 for the Science Writing Fellowships Program, and \$50,050 to provide scholarships to students in MBL courses. Donors' names are noted below. Those individuals who received fellowships and scholarships follow.

Robert Day Allen Fellowship

Drs. Jean M. and Joseph W. Sanger

American Society for Cell Biology Scholarships

Dr. Elizabeth Marincola, Executive Director

Frederik B. Bang Fellowship Fund

Mrs. Betsy G. Bang
Dr. Arthur M. Silverstein

**Frank A. Brown, Jr. Memorial
Readership**

Dr. Francis D. Carlson

The Charles R. Crane Fellowship

Friendship Fund

**The Jean and Katsuma Dan
Fellowship Fund**

Drs. Jean M. and Joseph W. Sanger
Mrs. Eleanor Steinbach

Bernard Davis Fund

Dr. Porter W. Anderson, Jr.
Dr. and Mrs. J. Woodland Hastings

Aline D. Gross Scholarship Fund

Mrs. Mona Gross
Mr. and Mrs. Alfred Weisberg

**Ann E. Kammer Memorial
Fellowship Fund**

Ms. Judy A. Akers

Stephen W. Kuffler Fellowship

Ms. Virginia Conquest
Dr. Thomas Oren Fox
Dr. Edward A. Kravitz
Prof. Brian M. Salzberg

Lakian Postdoctoral Scholar

Lakian Foundation

Lakian Summer Fellowships

Lakian Foundation

MBL Research Fellowships

Dr. and Mrs. Shinya Inoué
Dr. and Mrs. J. P. Trinkaus

Merck Scholarship Fund

Merck & Co., Inc.

**James A. and Faith Miller
Fellowship Fund**

Ms. Helen Cheney
Mr. J. Brad Coker
Mr. Stanley J. Kaiser
Dr. and Mrs. David A. Miller

Mountain Memorial Fund

Mr. and Mrs. Dean C. Allard, Jr.
Dr. Martha B. Baylor
Mr. Hugh C. Bell
Dr. William J. Beutler
Ms. Brenda J. Bodian
Ms. Elnor W. Bodian
Ms. Helen Bodian & Mr. Roger Alcala
Mr. G. Nathan Calkins, Jr.
Ms. Mildred S. Carson
Ms. Alice W. Cross
Mr. and Mrs. Brewster H. Gere, Jr.
Mr. and Mrs. R. G. Gillette
Mr. and Mrs. Alan J. Jacobsen
Col. William E. John
Mrs. Virginia Stokes Jones
Dr. and Mrs. Benjamin Kaminer
Ms. Anne C. Kimball
Mr. Kenneth H. Lange
Ms. Charlotte Z. LeMay
Mr. Harry Mellins
Mr. and Mrs. James E. Milligan
Ms. Helen T. North
Ms. Esther L. Racoosin
Reader's Digest Foundation
Mr. and Mrs. Thomas H. Roberts
Mr. Miles H. Robinson
Mr. and Mrs. Henry J. Rose
Mr. Ladislav Z. Sajor
Mr. William B. Sanford
Dr. and Mrs. R. Walter Schlesinger
Joel W. Silverstein, M.D.
Ms. Doris Small
Mr. and Mrs. Herbert G. Sparrow
Mr. James B. Steere
Mr. and Mrs. John W. Stewart
Dr. and Mrs. William N. Thomas

Nikon Fellowship

Nikon, Inc.

**Phillip H. Presley Memorial
Scholarships**

Carl Zeiss, Inc.

**Science Writing Fellowships
Program**

The American Association of Immunologists
American Society for Biochemistry and
Molecular Biology
American Society for Cell Biology
American Society for Investigative Pathology
American Society for Microbiology
American Society for Photobiology
American Society of Zoologists
Association for Research in Vision and
Ophthalmology
Association of Anatomy, Cell Biology and
Neurobiology Chairpersons
Biophysical Society
Charles A. Dana Foundation
Foundation for Microbiology
Friendship Fund
John S. and James L. Knight Foundation
Merck Company Foundation
New York Times Foundation
Nicholas B. Ottaway Foundation
The Washington Post Company

**The Moshe Shilo Memorial
Scholarship Fund**

Dr. and Mrs. J. Woodland Hastings
Dr. and Mrs. Laszlo Lorand

**The Evelyn and Melvin Spiegel
Fellowship Fund**

Dr. Paulo Alexandre Abrahamsohn
Dr. Michael Ovadia
Drs. Jean M. and Joseph W. Sanger
Drs. Evelyn and Melvin Spiegel

H. B. Steinbach Fellowship

Mrs. H. Burr Steinbach

**The Walter L. Wilson Endowed
Scholarship Fund**

Mrs. Marian Rigaumont
Dr. Jean R. Wilson

Young Scholars/Fellows Program

Mr. Gordon C. Estabrooks
Mrs. Rebeckah D. Glazebrook
Dr. Howard H. Hiatt
Mr. Robert A. Knudson
Dr. and Mrs. William M. McDermott

**Post-Course Research Support
Provided by**

Theodore D. Inoué
Carl Zeiss, Inc.

Fellowships Awarded

MBL Summer Research Fellows

- Ronald T. Aimes, the *Frederik B. Bang Fellow*, is a graduate student in the Department of Biochemistry at the State University of New York in Stony Brook, New York. His research focused on characterizing the receptor-associated protein (RAP) in extracts of *Limulus* amoebocyte that bind mammalian RAP thus establishing its homology to the protein in mammals.

- Jaume Baguña, Ph.D., the *James A. and Faith Miller Memorial Fund Fellow*, is Professor of Genetics at the University of Barcelona in Spain. Dr. Baguña studied cell lineage in early development in the marine flatworm, *Hoploplana inquilina*, one of the most primitive living animals with bilateral symmetry.

- Robert Créton, Ph.D., supported by the *Evelyn and Melvin Spiegel and the Eric F. Fries Fellowship Funds*, worked with Dr. Lionel Jaffe on directly measuring calcium levels in developing sea urchin embryos using the bioluminescent protein, aequorin, and the MBL's unique photon imaging system.

- Ana DePina, the *William Townsend Porter Fellow*, is a graduate student in the Department of Biology at Dartmouth College in New Hampshire. Ms. DePina investigated the mechanism by which organelles move along actin filaments using fluorescence, transmitted light, and electron microscopy.

- Leah Devlin, Ph.D., supported by the *MBL Associates and the MBL Research Fellowship Funds*, is an Assistant Professor of Biology at Penn State University in University Park, Pennsylvania. Dr. Devlin's research explored the function and evolution of invertebrate calcium channels and the role of extracellular calcium ions in the generation of cardiac and smooth muscle contractions.

- Paolo Gaudiano, Ph.D., supported by the *Esther A. and Joseph Klingenstein Fund and the M.G.F. Fuortes Fellowship Fund*, is an Assistant Professor in the Department of Cognitive and Neural Systems at Boston University in Massachusetts. Dr. Gaudiano investigated the behavioral bias of arousal-induced nonassociative learning as compared to operant conditioning in the head-waving behavior of the marine mollusc, *Aplysia californica*, based on the computational neural model that he recently devised.

- Jonathan Joseph Henry, Ph.D., supported by the *Lucy B. Lemann and the MBL Associates Fellowship Funds*, is an Assistant Professor in the Department of Cell and Structural Biology at the University of Illinois. Dr. Henry returned during the summer of 1995 to pursue his research on cell lineage of the nemertean, *Cerebratulus lacteus*.

- Diane Lipscombe, Ph.D., supported by the *H. Burr Steinbach and MBL Associates Fellowship Funds*, is an Assistant Professor in the Department of Neuroscience at Brown University in Rhode Island. Dr. Lipscombe's work was designed to determine how the electrical properties of sensory neurons are disrupted when the protein, neurofibromin, is not expressed.

- Kirk Malloy, Ph.D., supported by the *Frederik B. Bang and the MBL Associates Fellowship Funds*, is a Postdoctoral Fellow in the Biology Department at Northeastern University. Dr. Malloy investigated the developmental ontogeny of DNA repair and cell cycle control in *Fundulus* relative to processes of tumorigenesis caused or induced by exposure to ultraviolet radiation found in natural sunlight.

- Pierre Meyrand, Ph.D., a *Herbert W. Rand Fellow*, is an investigator in the Neurobiology and Comparative Physiology Laboratory at the University of Bordeaux in Arcachon, France. Dr. Meyrand studied the cellular mechanisms underlying development,

maturation and progressive differentiation of a single embryonic neural network towards the multiple functional networks in the adult.

- Lisa Moore, R.N., Ph.D., an *MBL Associates Fellow*, is a Postdoctoral Fellow in the Department of Neuroscience at Albert Einstein College of Medicine in New York. Dr. Moore studied the electrical synapses found between horizontal and amacrine cells that appear to play an important role in modulating visual acuity and light adaptation in the retina.

- John Murray, M.D., Ph.D., the *Nikon Fellow*, is Associate Professor in the Department of Cell and Developmental Biology at the University of Pennsylvania School of Medicine in Philadelphia, Pennsylvania. Dr. Murray used video DIC and fluorescence light microscopy to investigate individual microtubules derived from the mitotic spindle of sea urchins, *Arbacia*.

- Robert Palazzo, Ph.D., supported by the *Robert Day Allen and the MBL Research Fellowship Funds*, is Associate Professor in the Department of Physiology and Cell Biology at the University of Kansas in Lawrence, Kansas. Dr. Palazzo studied the centrosome, a subcellular organelle, which is integral to fundamental cell processes such as cell migration, replication, and maintenance of cell shape and polarity.

- Lucas Pozzo-Miller, Ph.D., a *Lakian Fellow*, is a Postdoctoral Fellow at the Roche Institute of Molecular Biology in Nutley, New Jersey. Dr. Pozzo-Miller investigated the first-order giant neurons in the axon of the squid, *Loligo pealei* L., using electrophysiological and ultrastructural methods.

- James Carl Precht, Ph.D., supported by the *MBL Associates and the MBL Research Fellowship Funds*, is Assistant Research Scientist in the Department of Neurosciences at the University of California, San Diego in La Jolla, California. Dr. Precht investigated spatiotemporal patterning related to visual stimulation in the pond turtle, *Pseudemys scripta*.

- Peter Reinhart, Ph.D., an *MBL Associates Fellow*, is Assistant Professor in the Department of Neurobiology at Duke University Medical Center in Durham, North Carolina. Dr. Reinhart investigated the role of ion channels in synaptic plasticity in Purkinje cells isolated from rat brain.

- Heidi Scrabble, Ph.D., supported by the *John O. Crane and the MBL Associates Fellowship Funds*, is Assistant Professor in the Department of Neuroscience at the University of Virginia in Charlottesville, Virginia. Dr. Scrabble's project was designed to characterize the functional defects in ion channels and their associated electrical currents that arise as a consequence of mutations at the NF1 locus using electrophysiological techniques.

- Kathleen King Siwicki, Ph.D., the *Frank R. Lillie Fellow*, is Assistant Professor in the Biology Department at Swarthmore College in Swarthmore, Pennsylvania. Dr. Siwicki studied the neurobiology and genetics of insect circadian rhythms in the fruit fly, *Drosophila melanogaster*, and in the hawkmoth, *Manduca sexta*, with emphasis on the role of the period (per) gene in controlling rhythmic behaviors.

- Joel Tabb, Ph.D., a *Lakian Fellow*, is Research Associate in the Department of Biology at Dartmouth College in New Hampshire. Dr. Tabb characterized endoplasmic reticulum-like, tubulovesicular organelles that move along actin filaments using fluorescent dyes and organelle-specific antibodies for immunoblotting and immunofluorescent microscopic studies.

- Phong Tran, the *Eric F. Fries Fellow*, is a graduate student in the Department of Biology at the University of North Carolina at Chapel Hill, North Carolina. Mr. Tran continued last summer's studies on the organization of microtubules within the mitotic spindle and kinetochore fibers during chromosome movement using the digital polarization microscope to obtain four-dimensional images.

- Anya Waite, Ph.D., the *Bernard Davis Fellow*, is a Postdoctoral Scholar in the Department of Biology at Woods Hole Oceanographic Institution. Ms. Waite investigated the physiological regulation of sugar-containing compounds on the surface of bloom-forming marine diatoms.

- James Walker, a *Herbert W. Rand Fellow*, is a post-graduate student in the Department of Biochemistry at the University of Cambridge in Cambridge, England. Mr. Walker investigated the mechanism of translational control of masked maternal mRNA in the early development of the clam, *Spisula solidissima*.

- James Zheng, Ph.D., supported by the *Stephen W. Kuffler and the MBL Associates Fellowship Funds*, is a Postdoctoral Fellow in Department of Biological Sciences at Columbia University in New York. Dr. Zheng studied how the growth cone at the tip of a neurite interacts with environmental signals and makes appropriate path-finding decisions to form specific neuronal connections.

Grass Fellows

- Miriam Ashley-Ross, Ph.D., University of California, Irvine. Project title: Work and power output of fish dorsal fin muscle.

- Thomas A. Cleland, University of California, San Diego. Project title: Glutamate-gated chloride channels of central pattern generating neurons in lobster stomatogastric ganglion.

- Victoria Pryor Connaughton, Ph.D., University of Texas at Houston. Project title: Modulation of ion channel activity by the actin cytoskeleton in teleost horizontal cells.

- William M. DeBello, Duke University Medical Center. Project title: Role of NSF in transmitter release at the squid giant synapse.

- Iñigo Novales Flamarique, University of Victoria, Canada. Project title: Optical studies of polarized light detection in fishes.

- Clifford Kentros, NYU School of Medicine. Project title: Evolution of thalamocortical circuitry.

- Peter Kloppenburg, Ph.D., University of Arizona. Project title: Voltage-activated currents in antennal motoneurons of the honey bee, *Apis mellifera*.

- Estela V. O'Brien, The Rockefeller University. Project title: Intrinsic optical signals of horseshoe crab brain—neural imaging techniques.

- Øystein Haug Olsen, Ph.D., Emory University. Project title: Mapping of interneuronal activity to motor neuron output in the heartbeat network of the medicinal leech.

- Kerry Quinn, Ph.D., University of Connecticut Health Science Center. Project title: Structure function relationship of the ryanidine receptor (RYR)/calcium release channel.

- Dejian Ren, SUNY, Buffalo. Project title: Functional studies of calcium channels modulated with targeted ribozymes against BETA subunit.

- Matt Wachowiak, The Whitney Laboratory, University of Florida. Project title: Central olfactory coding in a crustacean model system.

MBL Science Writing Fellows

Laura Beil, *Dallas Morning News*

David Berreby, *Freelance*

Jim Dawson, *Minneapolis Star Tribune*

William Dietrich, *Seattle Times*

Joshua Fischman, *Science* magazine

Vincent J. Kiernan, *New Scientist* magazine

Kim E. Motylewski, NPR's "Living on Earth"

Gary Robbins, *The Orange County Register*

John Schwartz, *The Washington Post*

James Shreeve, *Freelance*

Ruth SoRelle, *Houston Chronicle*

Traci Warson, *U.S. News & World Report*

Mark Wheeler, *Freelance*

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Andrea Morris, Princeton University

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The name by which the Corporation shall be known is
THE MARINE BIOLOGICAL LABORATORY

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirtieth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A. D. eighteen hundred and eighty-eight.

Charles Endicott
Commissioner of Corporations)

Articles of Amendment

(On File in the Office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meeting of the corporation.

Voted: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

"No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation

"Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.

"The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the bylaws of the corporation, require action by the Corporate Members "

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President, David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of \$10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

Paul Guzzi
Secretary of the Commonwealth)

Bylaws

(Revised August 7, 1992 and December 10, 1992)

ARTICLE I—THE CORPORATION

A. *Name and Purpose* The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and main-

tain a laboratory or station for scientific study and investigation and a school for instruction in biology and natural history.

B. *Nondiscrimination.* The Corporation shall not discriminate on the basis of age, religion, color, race, national or ethnic origin, sex or sexual preference in its policies on employment and administration or in its educational and other programs.

ARTICLE II—MEMBERSHIP

A. *Members.* The Members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees (the "Board"), upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by the Board. At any regular or special meeting of the Board, the Board may elect new Members. Members shall have no voting or other rights with respect to the Corporation or its activities except as specified in these Bylaws, and any Member may vote at any meeting of the Members in person only and not by proxy. Members shall serve until their death or resignation unless earlier removed with or without cause by the affirmative vote of two-thirds of the Trustees then in office. Any Member who has retired from his or her home institution may, upon written request to the Corporation, be designated a Life Member. Life Members shall not have the right to vote and shall not be assessed for dues.

B. *Meetings.* The annual meeting of the Members shall be held on the Friday following the first Tuesday in August of each year, at the Laboratory of the Corporation in Woods Hole, Massachusetts, at 9:30 a.m. The Chairperson of the Board shall preside at meetings of the Corporation. If no annual meeting is held in accordance with the foregoing provision, a special meeting may be held in lieu thereof with the same effect as the annual meeting, and in such case all references in these Bylaws, except in this Article II.B., to the annual meeting of the Members shall be deemed to refer to such special meeting. Members shall transact business as may properly come before the meeting. Special meetings of the Members may be called by the Chairperson or the Trustees, and shall be called by the Clerk, or in the case of the death, absence, incapacity or refusal by the Clerk, by any other officer, upon written application of Members representing at least ten percent of the smallest quorum of Members required for a vote upon any matter at the annual meeting of the Members, to be held at such time and place as may be designated.

C. *Quorum.* One hundred (100) Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person at a meeting attended by a quorum shall constitute action on behalf of the Members.

D. *Notice of Meetings.* Notice of any annual meeting or special meeting of Members, if necessary, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting at least 15 days before such meeting to each Member at his or her address as shown on the records of the Corporation.

E. *Waiver of Notice.* Whenever notice of a meeting is required to be given a Member, under any provision of the Articles or Organization or Bylaws of the Corporation, a written waiver thereof, executed before or after the Meeting by such Member, or his or her duly authorized attorney, shall be deemed equivalent to such notice.

F. *Adjournments.* Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present at the meeting, whether or not such Members constitute a quorum, or by any officer entitled to preside at or to act as Clerk of such meeting, if no Member is present or represented. It shall not be necessary to notify any Members of any adjournment unless no Member is present or represented at the meeting which is adjourned, in which case, notice of the adjournment shall be given in accordance with Article II.D. Any business which could have been transacted at any meeting of the Members as originally called may be transacted at an adjournment thereof.

ARTICLE III—ASSOCIATES OF THE CORPORATION

Associates of the Corporation. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees. The Associates of the Marine Biological Laboratory shall have no voting rights.

ARTICLE IV—BOARD OF TRUSTEES

A. *Powers.* The Board of Trustees shall have the control and management of the affairs of the Corporation. The Trustees shall elect a Chairperson of the Board who shall serve until his or her successor is elected and qualified. They shall annually elect a President of the Corporation. They shall annually elect a Vice

Chairperson of the Board who shall be Vice Chairperson of the meetings of the Corporation. They shall annually elect a Treasurer. They shall annually elect a Clerk, who shall be a resident of Massachusetts. They shall elect Trustees-at-Large as specified in this Article IV. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They shall choose such other officers and agents as they shall think best. They may fix the compensation of all officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board shall have the power to choose an Executive Committee from their own number as provided in Article V, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article V. They shall, from time to time, elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

B. *Composition and Election*

(1) The Board shall include 24 Trustees elected by the Board as provided below:

(a) At least six Trustees ("Corporate Trustees") shall be Members who are scientists, and the other Trustees ("Trustees-at-Large") shall be individuals who need not be Members or otherwise affiliated with the Corporation.

(b) The 24 elected Trustees shall be divided into four classes of six Trustees each, with one class to be elected each year to serve for a term of four years, and with each such class to include at least one Corporate Trustee. Such classes of Trustees shall be designated by the year of expiration of their respective terms.

(2) The Board shall also include the Chief Executive Officer, Treasurer and the Chairperson of the Science Council, who shall be *ex officio* voting members of the Board.

(3) Although Members or Trustees may recommend individuals for nomination as Trustees, nominations for Trustee elections shall be made by the Nominating Committee in its sole discretion. The Board may also elect Trustees who have not been nominated by the Nominating Committee.

C. *Eligibility.* A Corporate Trustee or a Trustee-at-Large who has been elected to an initial four-year term or remaining portion thereof, of which he/she has served at least two years, shall be eligible for re-election to a second four-year term, but shall be ineligible for re-election to any subsequent term until one year has elapsed after he/she has last served as a Trustee.

D. *Removal.* Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee to be so removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him or her.

E. *Vacancies.* Any vacancy in the Board may be filled by vote of a majority of the remaining Trustees present at a meeting of Trustees at which a quorum is present. Any vacancy in the Board resulting from the resignation or removal of a Corporate Trustee shall be filled by a Member who is a scientist.

F. *Meetings.* Meetings of the Board shall be held from time to time, not less frequently than twice annually, as determined by the Board. Special meetings of Trustees may be called by the Chairperson, or by any seven Trustees, to be held at such time and place as may be designated. The Chairperson of the Board, when present, shall preside over all meetings of the Trustees. Written notice shall be sent to a Trustee's usual or last known place of residence at least two weeks before the meeting. Notice of a meeting need not be given to any Trustee if a written waiver of notice executed by such Trustee before or after the meeting is filed with the records of the meeting, or if such Trustee shall attend the meeting without protesting prior thereto or at its commencement the lack of notice given to him or her.

G. *Quorum and Action by Trustees.* A majority of all Trustees then in office shall constitute a quorum. Any meeting of Trustees may be adjourned by vote of a majority of Trustees present, whether or not a quorum is present, and the meeting may be held as adjourned without further notice. When a quorum is present at any meeting of the Trustees, a majority of the Trustees present and voting (excluding abstentions) shall decide any question, including the election of officers, unless otherwise required by law, the Articles of Organization or these Bylaws.

H. *Transfers of Interests in Land.* There shall be no transfer of title nor long-term lease of real property held by the Corporation without prior approval of not less than two-thirds of the Trustees. Such real property transactions shall be finally acted upon at a meeting of the Board only if presented and discussed at a prior

meeting of the Board. Either meeting may be a special meeting and no less than four weeks shall elapse between the two meetings. Any property acquired by the Corporation after December 1, 1989 may be sold, any mortgage or pledge of real property (regardless of when acquired) to secure borrowings by the Corporation may be granted, and any transfer of title or interest in real property pursuant to the foreclosure or endorsement of any such mortgage or pledge of real property may be effected by any holder of a mortgage or pledge of real property of the Corporation, with the prior approval of not less than two-thirds of the Trustees (other than any Trustee or Trustees with a direct or indirect financial interest in the transaction being considered for approval) who are present at a regular or special meeting of the Board at which there is a quorum.

ARTICLE V—COMMITTEES

A. *Executive Committee* There shall be an Executive Committee of the Board of Trustees which shall consist of not more than eleven (11) Trustees, including *ex officio* Trustees, elected by the Board.

The Chairperson of the Board shall act as Chairperson of the Executive Committee and the Vice Chairperson as Vice Chairperson. The Executive Committee shall meet at such times and places and upon such notice and appoint such subcommittees as the Committee shall determine.

The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board except those powers specifically withheld, from time to time, by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may, from time to time, approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board.

The Executive Committee shall keep appropriate minutes of its meetings, which shall be reported to the Board. Any actions taken by the Executive Committee shall also be reported to the Board.

B. *Nominating Committee* There shall be a Nominating Committee which shall consist of not fewer than four nor more than six Trustees appointed by the Board in a manner which shall reflect the balance between Corporate Trustees and Trustees-at-Large on the Board. The Nominating Committee shall nominate persons for election as Corporate Trustees and Trustees-at-Large, Chairperson of the Board, Vice Chairperson of the Board, President, Treasurer, Clerk, Director of the Laboratory and such other officers, if any, as needed, in accordance with the requirements of these Bylaws. The Nominating Committee shall also be responsible for overseeing the training of new Trustees. The Chairperson of the Board of Trustees shall appoint the Chairperson of the Nominating Committee. The Chairperson of the Science Council shall be an *ex officio* voting member of the Nominating Committee.

C. *Science Council* There shall be a Science Council (the "Council") which shall consist of Members of the Corporation elected to the Council by vote of the Members of the Corporation, and which shall advise the Board with respect to matters concerning the Corporation's mission, its scientific and instructional endeavors, and the appointment and promotions of persons or committees with responsibility for matters requiring scientific expertise. Unless otherwise approved by a majority of the members of the Council, the Chairperson of the Council shall be elected annually by the Council. The chief executive officer of the Corporation shall be an *ex officio* voting member of the Council.

D. *Board of Overseers* There shall be a Board of Overseers which shall consist of not fewer than five nor more than eight scientists who have expertise concerning matters with which the Corporation is involved. Members of the Board of Overseers may or may not be Members of the Corporation and may be appointed by the Board of Trustees on the basis of recommendations submitted from scientists and scientific organizations or societies. The Board of Overseers shall be available to review and offer recommendations to the officers, Trustees and Science Council regarding scientific activities conducted or proposed by the Corporation and shall meet from time to time, not less frequently than annually, as determined by the Board of Trustees.

E. *Board Committees Generally* The Trustees may elect or appoint one or more other committees (including, but not limited to, an Investment Committee, a Development Committee, an Audit Committee, a Facilities and Capital Equipment Committee and a Long-Range Planning Committee) and may delegate to any such committee or committees any or all of their powers, except those which by law, the Articles of Organization or these Bylaws the Trustees are prohibited from delegating; provided that any committee to which the powers of the Trustees are delegated shall consist solely of Trustees. The members of any such committee shall have such tenure and duties as the Trustees shall determine. The Investment Committee, which shall oversee the management of the Corporation's endow-

ment funds and marketable securities shall include as *ex officio* members, the Chairperson of the Board, the Treasurer and the Chairperson of the Audit Committee, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business, but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

F. *Actions Without a Meeting* Any action required or permitted to be taken at any meeting of the Executive Committee or any other committee elected by the Trustees may be taken without a meeting if all members of such committees consent to the action in writing and such written consents are filed with the records of meetings. Members of the Executive Committee or any other committee elected by the Trustees may also participate in any meeting by means of a telephone conference call, or otherwise take action in such a manner as may, from time to time, be permitted by law.

G. *Manual of Procedures* The Board of Trustees, on the recommendation of the Executive Committee, shall establish guidelines and modifications thereof to be recorded in a Manual of Procedures. Guidelines shall establish procedures for: (1) Nomination and election of members of the Corporation, Board of Trustees and Executive Committee; (2) Election of Officers; (3) Formation and Function of Standing Committees.

ARTICLE VI—OFFICERS

A. *Enumeration* The officers of the Corporation shall consist of a President, a Treasurer and a Clerk, and such other officers having the powers of President, Treasurer and Clerk as the Board may determine, and a Director of the Laboratory. The Corporation may have such other officers and assistant officers as the Board may determine, including (without limitation) a Chairperson of the Board, Vice Chairperson and one or more Vice Presidents, Assistant Treasurers or Assistant Clerks. Any two or more offices may be held by the same person. The Chairperson and Vice Chairperson of the Board shall be elected by and from the Trustees, but other officers of the Corporation need not be Trustees or Members. If required by the Trustees, any officer shall give the Corporation a bond for the faithful performance of his or her duties in such amount and with such surety or sureties as shall be satisfactory to the Trustees.

B. *Tenure* Except as otherwise provided by law, by the Articles of Organization or by these Bylaws, the President, Treasurer, and all other officers shall hold office until the first meeting of the Board following the annual meeting of Members and thereafter, until his or her successor is chosen and qualified.

C. *Resignation* Any officer may resign by delivering his or her written resignation to the Corporation at its principal office or to the President or Clerk and such resignation shall be effective upon receipt unless it is specified to be effective at some other time or upon the happening of some other event.

D. *Removal* The Board may remove any officer with or without cause by a vote of a majority of the entire number of Trustees then in office, at a meeting of the Board called for that purpose and for which notice of the purpose thereof has been given, provided that an officer may be removed for cause only after having an opportunity to be heard by the Board at a meeting of the Board at which a quorum is personally present and voting.

E. *Vacancy* A vacancy in any office may be filled for the unexpired balance of the term by vote of a majority of the Trustees present at any meeting of Trustees at which a quorum is present or by written consent of all of the Trustees, if less than a quorum of Trustees shall remain in office.

F. *Chairperson* The Chairperson shall have such powers and duties as may be determined by the Board and, unless otherwise determined by the Board, shall serve in that capacity for a term coterminous with his or her term as Trustee.

G. *Vice Chairperson* The Vice Chairperson shall perform the duties and exercise the powers of the Chairperson in the absence or disability of the Chairperson, and shall perform such other duties and possess such other powers as may be determined by the Board. Unless otherwise determined by the Board, the Vice Chairperson shall serve for a one-year term.

H. *Director* The Director shall be the chief operating officer and, unless otherwise voted by the Trustees, the chief executive officer of the Corporation. The Director shall, subject to the direction of the Trustees, have general supervision of the Laboratory and control of the business of the Corporation. At the annual meeting, the Director shall submit a report of the operations of the Corporation for such year and a statement of its affairs, and shall, from time to time, report to the Board all matters within his or her knowledge which the interests of the Corporation may require to be brought to its notice.

I. *Deputy Director.* The Deputy Director, if any, or if there shall be more than one, the Deputy Directors in the order determined by the Trustees, shall, in the absence or disability of the Director, perform the duties and exercise the powers of the Director and shall perform such other duties and shall have such other powers as the Trustees may, from time to time, prescribe.

J. *President.* The President shall have the powers and duties as may be vested in him or her by the Board.

K. *Treasurer and Assistant Treasurer.* The Treasurer shall, subject to the direction of the Trustees, have general charge of the financial affairs of the Corporation, including its long-range financial planning, and shall cause to be kept accurate books of account. The Treasurer shall prepare a yearly report on the financial status of the Corporation to be delivered at the annual meeting. The Treasurer shall also prepare or oversee all filings required by the Commonwealth of Massachusetts, the Internal Revenue Service, or other Federal and State Agencies. The account of the Treasurer shall be audited annually by a certified public accountant.

The Assistant Treasurer, if any, or if there shall be more than one, the Assistant Treasurers in the order determined by the Trustees, shall, in the absence or disability of the Treasurer, perform the duties and exercise the powers of the Treasurer, shall perform such other duties and shall have such other powers as the Trustees may, from time to time, prescribe.

L. *Clerk and Assistant Clerk.* The Clerk shall be a resident of the Commonwealth of Massachusetts, unless the Corporation has designated a resident agent in the manner provided by law. The minutes or records of all meetings of the Trustees and Members shall be kept by the Clerk who shall record, upon the record books of the Corporation, minutes of the proceedings at such meetings. He or she shall have custody of the record books of the Corporation and shall have such other powers and shall perform such other duties as the Trustees may, from time to time, prescribe.

The Assistant Clerk, if any, or if there shall be more than one, the Assistant Clerks in the order determined by the Trustees, shall, in the absence or disability of the Clerk, perform the duties and exercise the powers of the Clerk and shall perform such other duties and shall have such other powers as the Trustees may, from time to time, prescribe.

In the absence of the Clerk and an Assistant Clerk from any meeting, a temporary Clerk shall be appointed at the meeting.

M. *Other Powers and Duties.* Each officer shall have in addition to the duties and powers specifically set forth in these Bylaws, such duties and powers as are customarily incident to his or her office, and such duties and powers as the Trustees may, from time to time, designate.

ARTICLE VII—AMENDMENTS

These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

ARTICLE VIII—INDEMNITY

Except as otherwise provided below, the Corporation shall, to the extent legally permissible, indemnify each person who is, or shall have been, a Trustee, director or officer of the Corporation or who is serving, or shall have served at the request of the Corporation as a Trustee, director or officer of another organization in which the Corporation directly or indirectly has any interest as a shareholder, creditor or otherwise, against all liabilities and expenses (including judgments, fines, penalties, and reasonable attorneys' fees and all amounts paid, other than to the Corporation or such other organization, in compromise or settlement) imposed upon or incurred by any such person in connection with, or arising out of, the defense or disposition of any action, suit or other proceeding, whether civil or criminal, in which he or she may be a defendant or with which he or she may be threatened or otherwise involved, directly or indirectly, by reason of his or her being or having been such a Trustee, director or officer.

The Corporation shall provide no indemnification with respect to any matter as to which any such Trustee, director or officer shall be finally adjudicated in such action, suit or proceeding not to have acted in good faith in the reasonable belief that his or her action was in the best interests of the Corporation. The Corporation shall provide no indemnification with respect to any matter settled or comprised unless such matter shall have been approved as in the best interests of the Corporation, after notice that indemnification is involved, by (i) a disinterested majority of the Board of the Executive Committee, or (ii) a majority of the Members.

Indemnification may include payment by the Corporation of expenses in defending a civil or criminal action or proceeding in advance of the final disposition of such action or proceeding upon receipt of an undertaking by the person indemnified to repay such payment if it is ultimately determined that such person is not entitled to indemnification under the provisions of this Article VIII, or under any applicable law.

As used in the Article VIII, the terms "Trustee," "director," and "officer" include their respective heirs, executors, administrators and legal representatives, and an "interested" Trustee, director or officer is one against whom in such capacity the proceeding in question or another proceeding on the same or similar grounds is then pending.

To assure indemnification under this Article VIII of all persons who are determined by the Corporation or otherwise to be or to have been "fiduciaries" of any employee benefits plan of the Corporation which may exist, from time to time, this Article VIII shall be interpreted as follows: (i) "another organization" shall be deemed to include such an employee benefit plan, including without limitation, any plan of the Corporation which is governed by the Act of Congress entitled "Employee Retirement Income Security Act of 1974," as amended, from time to time, ("ERISA"); (ii) "Trustee" shall be deemed to include any person requested by the Corporation to serve as such for an employee benefit plan where the performance by such person of his or her duties to the Corporation also imposes duties on, or otherwise involves services by, such person to the plan or participants or beneficiaries of the plan; (iii) "lines" shall be deemed to include any excise tax plan pursuant to ERISA; and (iv) actions taken or omitted by a person with respect to an employee benefit plan in the performance of such person's duties for a purpose reasonably believed by such person to be in the interest of the participants and beneficiaries of the plan shall be deemed to be for a purpose which is in the best interests of the Corporation.

The right of indemnification provided in this Article VIII shall not be exclusive of or affect any other rights to which any Trustee, director or officer may be entitled under any agreement, statute, vote of Members or otherwise. The Corporation's obligation to provide indemnification under this Article VIII shall be offset to the extent of any other source of indemnification of any otherwise applicable insurance coverage under a policy maintained by the Corporation or any other person. Nothing contained in the Article shall affect any rights to which employees and corporate personnel other than Trustees, directors or officers may be entitled by contract, by vote of the Board or of the Executive Committee or otherwise.

ARTICLE IX—DISSOLUTION

The consent of every Trustee shall be necessary to effect a dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such a manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Trustees then in office in accordance with the laws of the Commonwealth of Massachusetts.

ARTICLE X—MISCELLANEOUS PROVISIONS

A. *Fiscal Year.* Except as otherwise determined by the Trustees, the fiscal year of the Corporation shall end on December 31st of each year.

B. *Seal.* Unless otherwise determined by the Trustees, the Corporation may have a seal in such form as the Trustees may determine, from time to time.

C. *Execution of Instruments.* All checks, deeds, leases, transfers, contracts, bonds, notes and other obligations authorized to be executed by an officer of the Corporation in its behalf shall be signed by the Director or the Treasurer except as the Trustees may generally or in particular cases otherwise determine. A certificate by the Clerk or an Assistant Clerk, or a temporary Clerk, as to any action taken by the Members, Board of Trustees or any officer or representative of the Corporation shall as to all persons who rely thereon in good faith be conclusive evidence of such action.

D. *Corporate Records.* The original, or attested copies, of the Articles of Organization, Bylaws and records of all meetings of the Members shall be kept in

Massachusetts at the principal office of the Corporation, or at an office of the Corporation's Clerk or resident agent. Said copies and records need not all be kept in the same office. They shall be available at all reasonable times for inspection by any Member for any proper purpose, but not to secure a list of Members for a purpose other than in the interest of the applicant, as a Member, relative to the affairs of the Corporation.

E. *Articles of Organization* All references in these Bylaws to the Articles of Organization shall be deemed to refer to the Articles of Organization of the Corporation, as amended and in effect, from time to time.

F. *Transactions with Interested Parties*. In the absence of fraud, no contract or other transaction between this Corporation and any other corporation or any firm, association, partnership or person shall be affected or invalidated by the fact that any Trustee or officer of this Corporation is pecuniarily or otherwise interested in or is a director, member or officer of such other corporation or of such firm, association or partnership or in a party to or is pecuniarily or otherwise

interested in such contract or other transaction or is in any way connected with any person or person, firm, association, partnership, or corporation pecuniarily or otherwise interested therein; provided that the fact that he or she individually or as a director, member or officer of such corporation, firm, association or partnership in such a party or is so interested shall be disclosed to or shall have been known by the Board of Trustees or a majority of such Members thereof as shall be present at a meeting of the Board of Trustees at which action upon any such contract or transaction shall be taken; any Trustee may be counted in determining the existence of a quorum and may vote at any meeting of the Board of Trustees for the purpose of authorizing any such contract or transaction with like force and effect as if he/she were not so interested, or were not a director, member or officer of such other corporation, firm, association or partnership, provided that any vote with respect to such contract or transaction must be adopted by a majority of the Trustees then in office who have no interest in such contract or transaction.

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- Annual Report of the Marine Biological Laboratory** R1

Volume 191

Number 2

THE BIOLOGICAL BULLETIN



OCTOBER, 1996

Published by the Marine Biological Laboratory

FEB 25 1997

THE BIOLOGICAL BULLETIN

PUBLISHED BY
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OCTOBER, 1996

Printed and Issued by
LANCASTER PRESS, Inc.

3575 HEMPLAND ROAD
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THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to Subscription Manager, THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543. Single numbers, \$38.00. Subscription per volume (three issues), \$95.00 (\$190.00 per year for six issues).

Communications relative to manuscripts should be sent to Michael J. Greenberg, Editor-in-Chief, or Pamela L. Clapp, Managing Editor, at the Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543. Telephone: (508) 289-7428, FAX: 508-457-1924. E-mail: pclapp@mbi.edu.

THE BIOLOGICAL BULLETIN is indexed in bibliographic services including *Index Medicus* and MEDLINE, *Chemical Abstracts*, *Current Contents*, and *CABS (Current Awareness in Biological Sciences)*.

Printed on acid free paper,
effective with Volume 180, Issue 1, 1991.

POSTMASTER: Send address changes to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory,
7 MBL Street, Woods Hole, MA 02543.

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Periodicals postage paid at Woods Hole, MA, and additional mailing offices.
ISSN 0006-3185

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Complete Interference and Nonrandom Distribution of Meiotic Crossover in a Mollusc, *Mulinia lateralis* (Say)

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*In gene mapping, the genetic distance between two genes is measured by the frequency of meiotic crossovers occurring between them. It is generally assumed that there is more than one crossover per chromosome, and the distribution of crossovers along chromosomes is random and follows a Poisson distribution (no interference), or that interference is inversely correlated with distance. Although those assumptions may be correct for some organisms, we report here a novel exception in the marine mollusc *Mulinia lateralis* Say. Using segregation analysis of gynogenetic diploids, we found surprisingly high gene-centromere recombinant frequencies for most of the 13 allozyme loci studied. For at least six loci, there was always one and only one crossover occurring between the gene and its centromere, suggesting complete interference where the occurrence of one crossover completely suppressed the occurrence of another. The complete interference was confirmed by the cytogenetic observation that there was only one chiasma for all bivalents. Further, sites of the single crossover seem not to be randomly distributed along chromosomes, but preferentially located in a recombination hot-region proximal to the centromere. The restricted distribution of a single crossover per chromosome provides one explanation for the unique phenomenon of heterozygote deficiency in *M. lateralis* and other molluscs.*

During meiosis, homologous chromosomes—one maternal and one paternal—duplicate and pair up, forming a bivalent consisting of four chromatids. Genetic recombination occurs when two homologous chromatids cross over. Meiotic crossovers are thought to occur randomly

along the chromosome, and their frequencies are correlated with physical DNA distances. In gene mapping, therefore, the frequency of crossovers (or recombinants) is used to estimate genetic distance. Gene mapping becomes complicated under crossover interference, where the occurrence of one crossover affects the chance of another one occurring nearby. Although the importance of modeling crossover interference has recently been recognized in gene mapping (1–3), our basic understanding of meiotic crossover and interference remains limited.

Gene-centromere recombination analysis provides a unique opportunity to study meiotic crossover. Because every gene is known to have linkage to a centromere, gene-centromere crossover can be reliably analyzed over a long distance. Linkage analysis of two genes, on the other hand, is difficult across long distance. Gene-centromere mapping is possible in a few species of fungi and algae through the analysis of unseparated meiotic products such as tetrads (4). In organisms such as fish and molluscs, viable gynogenetic diploids, equivalent to half-tetrads, can be produced and used for the purpose of gene-centromere mapping (5–7). Previously, we produced meiotic gynogens in *M. lateralis* by inhibiting the second meiotic division in gynogenetically activated eggs (8). In this study, the gynogenetic diploids along with their triploid controls were used to investigate gene-centromere recombination of allozyme loci.

The success of gynogenesis was determined by the absence of paternal alleles in gynogenetic offspring. In all gynogens analyzed from three families, none of the diagnostic paternal alleles (8 to 11 per family) were found, suggesting that gynogenesis was complete. In normal diploids and triploids, paternal alleles were present as expected from Mendelian segregation.

Thirteen allozyme loci were heterozygous in maternal parents, and their centromere recombinant frequencies (CR) were determined as the percentage of maternal heterozygotes among gynogenetic and triploid offspring. Segregation data from triploids were used only for loci whose paternal contribution could be clearly distinguished. Surprisingly high CR values were observed for most loci (Table I). The high CR values (or proportion of heterozygotes) cannot be explained by reduced survival of gynogens homozygous for recessive lethal genes. Triploids, which should be unaffected by recessive lethals, gave almost identical CR estimates as gynogens in all cases. Therefore, the high CR values must be a true reflection of high crossover frequencies between allozyme loci and their centromeres. A CR value of 100% means that there was always one and only one crossover occurring between the gene and its centromere, which is complete interference by definition; *i.e.*, the occurrence of the one crossover completely inhibits the occurrence of another one. Among the 13 loci, at least six, AAT, ALAT2, GPI, EST, MPI, and PGDH, exhibited complete interference. Another four loci, ALAT1, LAP, PGM, and TAP3, had high levels of interference with CR values higher than 95%, although complete interference could not be ruled out. Complete interference for all chromosomes is confirmed by cytogenetic data. *M. lateralis* has a haploid number of 19 chromosomes, all of which are telocentric (8, 11). For 16 meioses examined in eggs, all 304 (16×19) synapsed bivalents had a single chiasma (Fig. 1). Previously published photographs of meiotic chromosomes also clearly demonstrated one chiasma per bivalent (11). Although positive interference has been considered the rule for eukaryotes, most species have more than one crossover per bivalent and only moderate levels of interference; complete interference is rare (12–14). The telocentric nature of *M. lateralis* chromosomes is probably not the reason for only one chiasma per chromosome. Telocentric chromosomes of mice frequently have two chiasmata per chromosome (15). Because all parents were randomly selected from one base population, crossover inhibition by polymorphic inversions was also unlikely in this study.

Knowing the level of interference is crucial to the accuracy of gene mapping. In mapping analysis, it is generally assumed that there is more than one crossover per chromosome, and the distribution of crossovers is random and follows a Poisson distribution (no interference), or that interference is inversely correlated with genetic distance (16, 17). When there is complete or high levels of interference over the whole chromosome, as suggested by this study, those assumptions become invalid, and most models of map functions will inflate genetic distances. Under complete interference in *M. lateralis*, for example, a genetic distance of 20 cM would be inflated

Table I

Gene-centromere recombinant frequency (CR) of 13 allozyme loci estimated from gynogenetic diploid (Gyno) and triploid (3n) *Mulinia lateralis*

Loci		Offspring Genotype			CR (%)	Distance (cM)
		AA	AB	BB		
AH2	Gyno	101	1	75	1.3	0.6
	3n	26	2	27		
AH1	Total	127	3	102	51.4	25.7
	Gyno	15	27	13		
DAP	3n	17	29	8	69.5	34.7
	Total	32	56	21		
PGM	Gyno	18	82	18	95.6	47.8
	3n	3	111	3		
ALAT1	Total	6	216	4	96.0	48.0
	Gyno	3	169	4		
TAP3	3n	1	168	3	97.6	48.8
	Total	2	246	4		
LAP	Gyno	1	117	0	99.4	49.7
	3n	0	55	0		
AAT	Total	1	172	0	100.0	50.0
	Gyno	0	118	0		
ALAT2	3n	0	71	0	100.0	50.0
	Total	0	189	0		
GPI	Gyno	0	117	0	100.0	50.0
	3n	0	55	0		
EST	Total	0	172	0	100.0	50.0
	Gyno	0	119	0		
MPI	3n	0	52	0	100.0	50.0
	Total	0	171	0		
PGDH	Gyno	0	178	0	100.0	50.0
	3n	0	106	0		
	Total	0	284	0	100.0	50.0
	Gyno	0	118	0		
	3n	0	109	0		
	Total	0	227	0	100.0	50.0

Gene-centromere recombinant frequency (CR) was calculated as the portion of maternal heterozygotes among offspring (6, 7). CR estimates from gynogens and triploids of three families were combined for each locus because they did not differ significantly. Genetic distance was calculated by halving the CR values under the assumption of complete interference (4). Allozyme analysis of adult clams was conducted by starch gel electrophoresis (9, 10). Names of the allozymic loci were abbreviated as: AAT for aspartate aminotransferase (E.C. 2.6.1.1), AH for aconitate hydratase (E.C. 4.2.1.3), ALAT for alanine aminotransferase (E.C. 2.6.1.2), DAP for dipeptidase (E.C. 3.4.*.*Gly-Leu as substrate), EST for esterase (E.C. 3.1.1.1), GPI for glucose-6-phosphate isomerase (E.C. 5.3.1.9), LAP for leucine aminopeptidase (E.C. 3.4.*.*), MPI for mannose-6-phosphate isomerase (E.C. 5.3.1.8), PGDH for phosphogluconate dehydrogenase (E.C. 1.1.1.44), PGM for phosphoglucomutase (E.C. 5.4.2.2), TAP for tripeptidase (E.C. 3.4.*.*Leu-Gly-Gly as substrate). AAT, ALAT2 and GPI were closely linked without double-crossover occurring among them (Guo *et al.*, in prep).

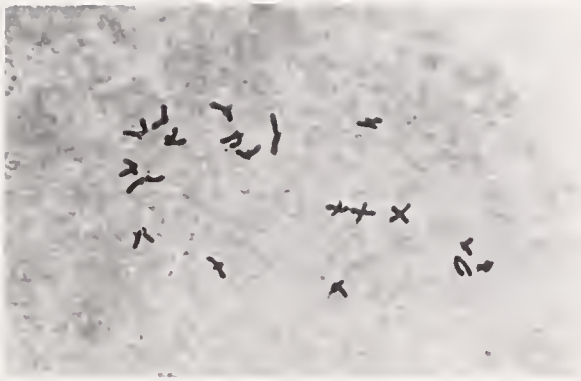


Figure 1. Chiasma formation in eggs of the dwarf surfclam, *Mulinia lateralis*, showing "V" or "X" shaped bivalents with only one chiasma. If there were two or three crossovers per chromosome, bivalents would have appeared in the shapes of "O" or "8" respectively. The single chiasma usually formed near the centromere ("V" shaped), except for 2 or 3 bivalents. Eggs were fixed in 3:1 methanol and acetic acid and stained with an acetic orcein stain.

by 6% with Kosambi's map function and by 28% with Haldane's function, and the inflation increases dramatically for longer distances.

The agreement between the finding of one crossover per chromosome through segregation analysis (at six loci) and the cytogenetic observation of one chiasma per chromosome weakens the chiasma reduction hypothesis. The chiasma reduction hypothesis states that some chiasmata may be eliminated prior to metaphase, and the observed chiasma frequency is an underestimate of actual recombination events, causing the chiasma-based maps to be shorter than recombination-based maps (18, 19). Our results indicate that there was no chiasma reduction—one recombination corresponds directly to one chiasma—for at least four of the 19 chromosomes in *M. lateralis* (AAT, GPI, ALAT2 are linked). For four other chromosomes, chiasma reduction, if any, must be small, because the chance that two recombinations occurred is less than 5% according to the CR values. It is true that recombination maps are usually longer than chiasma maps (18, 20). This discrepancy is likely caused by the inflation of the linkage map due to the underestimation of interference as discussed earlier, and not by chiasma reduction.

Another unexpected finding was that the CR values of the 13 loci seem not to be randomly distributed between 0 and 100 (Table I). Ten of the 13 loci (77%) had a CR of 95 or higher; and 12 of the 13 loci (92%) had a CR of 50 or higher. In terms of genetic distance, most of the loci appeared to be located near the ends of chromosomes: 77% of the loci in a distal region, which accounts for only 5% of the chromosomal length, if we could assume that the distribution of crossovers is random. A casual exam-

ination of the mouse and human genetic maps reveals no restricted distribution of allozyme genes (21, 22). Another possibility is that the allozyme loci are randomly distributed along chromosomes, but the crossover sites are not. The nonrandom distribution of crossover sites is supported by cytogenetic observations. Chiasmata indeed tend to form near the centromeres in eggs of *M. lateralis* and produce "V" shaped bivalents, evident in Figure 1 as well as in previously published photographs of meiotic chromosomes (11). Therefore, our interpretation of both the recombination and chiasma data is that crossover sites in female *M. lateralis* are preferentially distributed in a recombination hot-region proximal to the centromere. At the molecular level, recombination does not occur randomly at any place along the DNA, and recombination sites (often referred to as hotspots) are determined by particular sequences, such as the Chi (GCTGGTGG) in *E. coli*, (AGGC)_n in mouse, and the minisatellite sequences in mammals (23–25). However, the recombination hotspots identified so far account for only a small percentage of the total recombination events. The recombination hot-regions, as suggested by this study, could be chromosomal regions where the recombination hotspots are densely populated, or activated. It is not known whether the recombination hot-regions are related to heterochromatic regions.

Normally the nonrandom distribution of crossovers (or chiasmata) occurs where there are two or more chiasmata per chromosome, so that the position of the first chiasma affects the position of another one (chiasma interference). This study indicates that, even when there is only one chiasma per chromosome, this chiasma is still not randomly distributed along chromosomes. Most models for mechanisms of chiasma determination are based on chiasma interaction, including the most prevailing one—Fox's sequential model (12, 13). According to the sequential model, chiasma formation is sequential from telomere to centromere, and when there is only one chiasma, it is usually located near the telomere. In *M. lateralis*, the single chiasma is near the centromere.

The finding of only one crossover per chromosome with restricted distribution may explain the high levels of heterozygosity in *M. lateralis* and other marine bivalves (compared with most other animals), and the unique phenomenon of heterozygote deficiency (26, 27). In chromosomal regions having few crossovers, linkage disequilibrium would interfere with selection, resulting in the coexistence of numerous alleles at a locus. For example, if a deleterious allele at one locus is linked to a favorable allele at another, and the linkage is strong due to a lack of recombination, the deleterious allele could not be easily eliminated by selection, leading to high levels of heterozygosity and possibly heavy genetic loads. Further, genes within the linkage group could become coadapted

as a block, the survival of which would no longer be determined by the fitness of individual alleles, but would be dependent on encountering a similar, complementary, or coevolved block. If two blocks differed too much, they might produce heterozygotes which would be less fit than homozygotes. The coadapted complex may provide one explanation for heterozygote deficiency, but many other hypotheses have previously been advanced (27, 28, 29).

Acknowledgments

We thank S. F. Lyu and G. Denham for help with electrophoretic analysis and P. M. Gaffney and anonymous reviewers for comments. This work was partly supported by grant #92-37703-7766 from the US Department of Agriculture/NRICGP. Publication No. D-32100-2-96, NJAES.

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An Ancient Chemosensory Mechanism Brings New Life to Coral Reefs

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The first scleractinians, progenitors of modern corals, began to appear 240 million years ago; by the late Jurassic (150 Ma) most families of modern corals had evolved and begun forming reefs (1, 2). Mechanisms controlling the recruitment of new corals to sustain these structures are, however, poorly understood (3). Corals, like many marine invertebrates, begin life as soft-bodied larvae that are dispersed in the plankton (3, 4). As the first step in developing a calcified coral colony, the larva must settle out of the plankton onto a suitable substratum and metamorphose to the single calcified polyp stage cemented to the reef (3, 5). Our analyses of the metamorphic requirements of larvae in divergent coral families surprised us by revealing the existence of a common chemosensory mechanism that is required to bring larvae out of the plankton and onto the reef. This mechanism appears to be quite old, predating both the phylogenetic divergence of these coral families and the development of different modes of coral reproduction.

We analyzed the requirements for metamorphosis of larvae from 10 species of Pacific *Acropora*—the acroporid genus with by far the greatest number of known species (76 in the Indo-Pacific, about one-sixth the estimated number of scleractinian species in that region)—and three species representing three common genera of Pacific Faviidae, the second most speciose scleractinian family (1, 2). Most, but not all, species of *Acropora* and *Favia* (genera that first appear in the fossil record in the Eocene and Cretaceous, respectively) are widely distrib-

uted throughout the Indo-Pacific; *Cyphastrea* and *Goniastrea* (first appearing in the Oligocene and Eocene, respectively), although common, are limited to the Indo-Pacific (1, 2). Larvae of the acroporids and faviids that we tested are generated by cross-fertilization of gametes released into the plankton during mass-spawning events, the dominant form of sexual reproduction in corals (3). We found that larval detection of suitable reef substrata is controlled by chemosensory recognition of a cue associated with encrusting red algae, among the major cementers of the reef. A similar process operates in Caribbean agariciid species that brood their larvae (5–10). These agariciids are in two genera, *Agaricia* and *Leptoseris*, that first appeared in the Miocene and Oligocene, respectively (1, 2). Our findings thus suggest that this chemosensory mechanism is common to at least the Acroporidae, Faviidae, and Agariciidae—three major and divergent coral families.

In the laboratory at Akajima, Japan, larvae of the widely distributed mass-spawning Indo-Pacific corals *Acropora nasuta* and *A. digitifera* exhibit a strict requirement for a specific environmental cue: surface contact with sympatric crustose red algae is required for cue detection (Fig. 1A, B). The strength of the larval response (% metamorphosis) to all five crustose red algae tested varies directly with larval age (days post-fertilization). But regardless of larval age, the response to the seawater and brown algal controls remains nil (0% metamorphosis). Overall, *A. digitifera* was significantly more responsive to inductive algae than was *A. nasuta* (two-way ANOVA; coral effect: $F = 18.3$, $df = 1$, $P = 0.0016$). Although there was no significant difference between species in their response to these algae (two-way AN-

Received 28 May 1996; accepted 7 August 1996.

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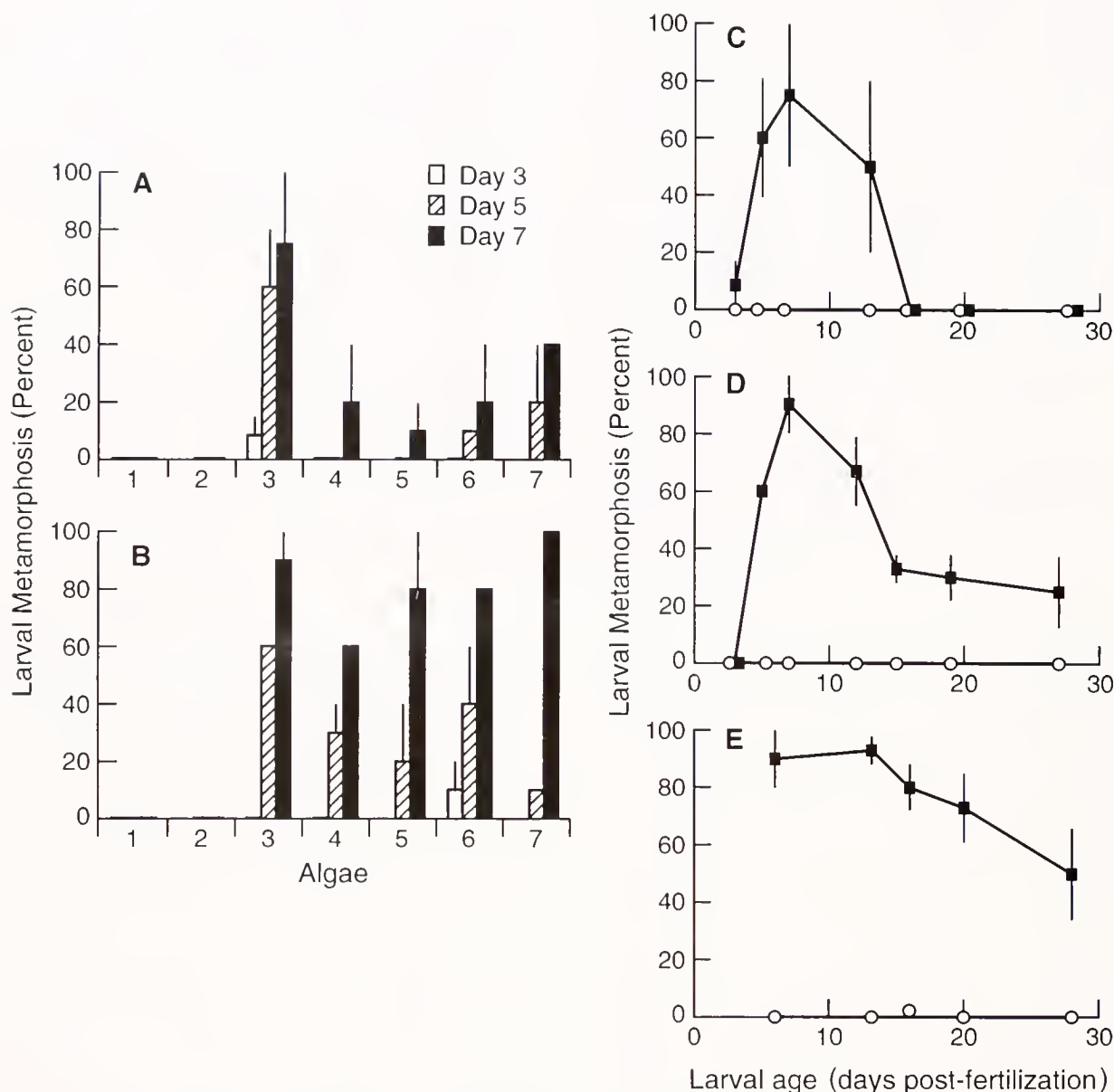


Figure 1. Pacific acroporid larvae exhibit a stringent requirement for contact with crustose red algae for metamorphosis; development and decay of metamorphic competence is dependent on larval age. (A) Metamorphosis of *Acropora nasuta* larvae (3, 5 and 7 days post-fertilization) in response to various crustose red algae and controls after 48 h incubation in 10 ml filtered seawater (FSW). Algae: 1 = 0.22- μ m FSW alone (control); 2 = *Lobophora variegata* (brown alga, control); 3 = *Peyssonnelia* sp.; 4 = *Hydrolithon reinboldii* (form i); 5 = *Hydrolithon reinboldii* (form ii); 6 = *Hydrolithon reinboldii* (form iii); 7 = *Hydrolithon reinboldii* (form iv). Values are mean $\pm$ (1 std. error) metamorphosis of five individuals in each of two trials ($n = 2$). Percentages were arcsine transformed for analysis. (B) Metamorphic response of *Acropora digitifera* larvae (3, 5 and 7 days post-fertilization) to algae and controls, as in (A). (C) Metamorphosis of *A. nasuta* larvae after a 48-h incubation with *Peyssonnelia* sp. (■) or a parallel exposure to FSW alone (○). Individual points represent ages (days post-fertilization) at challenge; metamorphosis (%) = mean; error bars = s.e. of $n = 2$ (days 3–7 or 3 (days > 7)). (D) *A. digitifera* larval response as for (C). (E) *A. tenuis* larval response as for (C). $n = 3$ all days. Larvae of each species were obtained as follows: The maturity of coral gonads was confirmed (14); colonies were transferred from the reef to laboratory aquaria and kept in darkness until the gamete clusters were ready to be released; the colonies were transferred to individual containers for spawning; gamete clusters from a single colony were collected and dispersed by sequential transfer into FSW; eggs and sperm were separated for immediate cross-fertilization with gametes of other colonies ($\geq 95\%$ fertilization rate); all spawnings (1/species) produced 5–10,000 larvae/batch; each batch was cultured in 3 l FSW (changed daily) at 26°C for ≥ 30 d; larvae of each species were held in these rearing chambers until randomly selected for assay at the ages indicated. For assays, fragments were

OVA; coral * algae interaction: $F = 0.89$, $df = 4$, $P = 0.503$), *A. digitifera* exhibited less variation in response among algae (one-way ANOVA: $F = 0.61$, $df = 6$, $P = 0.72$) than did *A. nasuta* (one-way ANOVA: $F = 2.9$, $df = 6$, $P = 0.095$). Such patterns are similar to the species-specific differences previously found in agariciid corals (10).

Larvae maintained in seawater alone (≥ 30 days) continue planktonic swimming and never develop beyond the larval stage illustrated in Fig. 2A (Fig. 1C, D, E). Metamorphosis is initiated on contact with an inductive alga, e.g., *Peyssonnelia* sp. or any of four morphological forms of *Hydrolithon reinboldii*; larvae rapidly stop swimming, their bodies elongate and remain in close contact with the algal surface, and within a few hours, they round up and cement themselves to the algal surface or adjacent substratum. The final stages of metamorphosis are marked by the development of 12 radial skeletal elements, the calcified septa and costae (Fig. 2B). Larvae of the mass-spawning congeners *A. tenuis*, *A. florida*, *A. gemmifera*, *A. formosa*, *A. hyacinthus*, *A. sp. 1*, *A. sp. 4* and *A. sp. 5* all exhibit a similar strict requirement for contact with either or both *Peyssonnelia* sp. and *H. reinboldii* (Fig. 2C; and A. N. C. Morse *et al.*, University of California, Santa Barbara, in prep). Although, in some instances, the brown alga *Lobophora variegata* promoted first stage elongation of the larvae, further development rarely if ever occurred. When larvae from seven acroporid species (50 larvae/species incubated together) were given a choice between crustose red algae and brown algae, all that had metamorphosed after 4-h exposure (70%) were found only on the crustose red algae (*Peyssonnelia* sp. and *H. reinboldii*) (Fig. 2C); no larval metamorphosis was detected on brown algae or on the chamber surfaces; control larvae (same number and species) remained swimming. In a similar experiment, 500 competent larvae from each of five acroporid species (*A. nasuta*, *A. tenuis*, *A. formosa*, *A. hyacinthus*, and *A. gemmifera*, tested together in a 30-l flow-through tank) were presented a choice of algae, live corals, inert substrata collected from the reef, "fouled" panels, and a variety of inert materials commonly used as settlement plates; all settlement and metamorphosis occurred only on coralline algae (A. N. C. Morse *et al.*, University of California, Santa Barbara, in prep.).

The stringency of this requirement (no metamorphosis in the absence of an exogenous cue) and the specificity

(dependence on crustose red algae) of cue recognition persist for the duration of larval competence for metamorphosis (Fig. 1C, D, E). Species-specific differences in the duration of competence are obvious. The magnitude of the larval response for each species is dependent upon larval age (Fig. 1). Larvae exhibited little or no response to *Peyssonnelia* sp. at 3 days post-fertilization; responsiveness developed by 5 days, peaked at 7 days, and declined thereafter at rates that are species-specific (Fig. 1C, D, E).

If these results reflect larval potential for dispersal in the plankton, then the differences in the rates of decline of competence among all of the tested acroporid species, with no accompanying loss in stringency or specificity of cue requirement (e.g., Fig. 1), suggest species-specific differences in the windows of opportunity for successful settlement and metamorphosis on the reef. Assuming that rate of decrease of competence with larval age is inversely correlated with the distance of dispersal from parental colonies (all other chemical and hydrodynamic factors being equal), *A. tenuis* appears to have the potential for widest dispersal and significant recruitment for at least 5–30 days post-fertilization. *A. digitifera* appears to have the potential for somewhat wider dispersal than *A. nasuta*, although the majority of potential recruits from both species will be dispersed over similar distances (5–12 days). Although the bulk of settlement and metamorphosis might occur soon after the larvae reach competence, these differences among species that recognize the same algae may serve to decrease post-settlement competition for space among some of the settlers. Variation among these species in timing of gamete release during mass-spawning events (11–14), in concert with variations in currents and other hydrodynamic factors, may additionally contribute to a reduction in the potential for post-settlement interaction.

Significantly, we believe, larvae of these Pacific acroporids and those in two Caribbean genera, *Agaricia* and *Leptoseris* (Agariciidae), both appear to recognize and require the same class of algal cue for the induction of larval settlement and metamorphosis. This is in spite of their very different modes of sexual reproduction and larval development (3). Acroporids participate in mass-spawning events during which millions of gametes are released into the plankton for cross-fertilization, followed by larval development in the plankton; in contrast, agariciids have evolved the less common mode of

prepared from algae as previously described (6, 7, 9); live *H. reinboldii* specimens were grouped according to the surface characteristics of different growth forms (by A. N. C. M.); specimens preserved in formalin and seawater were dehydrated, embedded in paraffin, sectioned (8- μ m thickness), and identified with a light microscope to species and genus level (by M. B.)—*Lobophora variegata* (Lamouroux), *Hydrolithon reinboldii* (Weber van Bosse et Foslief) Foslief; *Peyssonnelia* sp. [similar to *P. obscura* Weber van Bosse and *P. conchicola* Piccone et Grunow (19)].

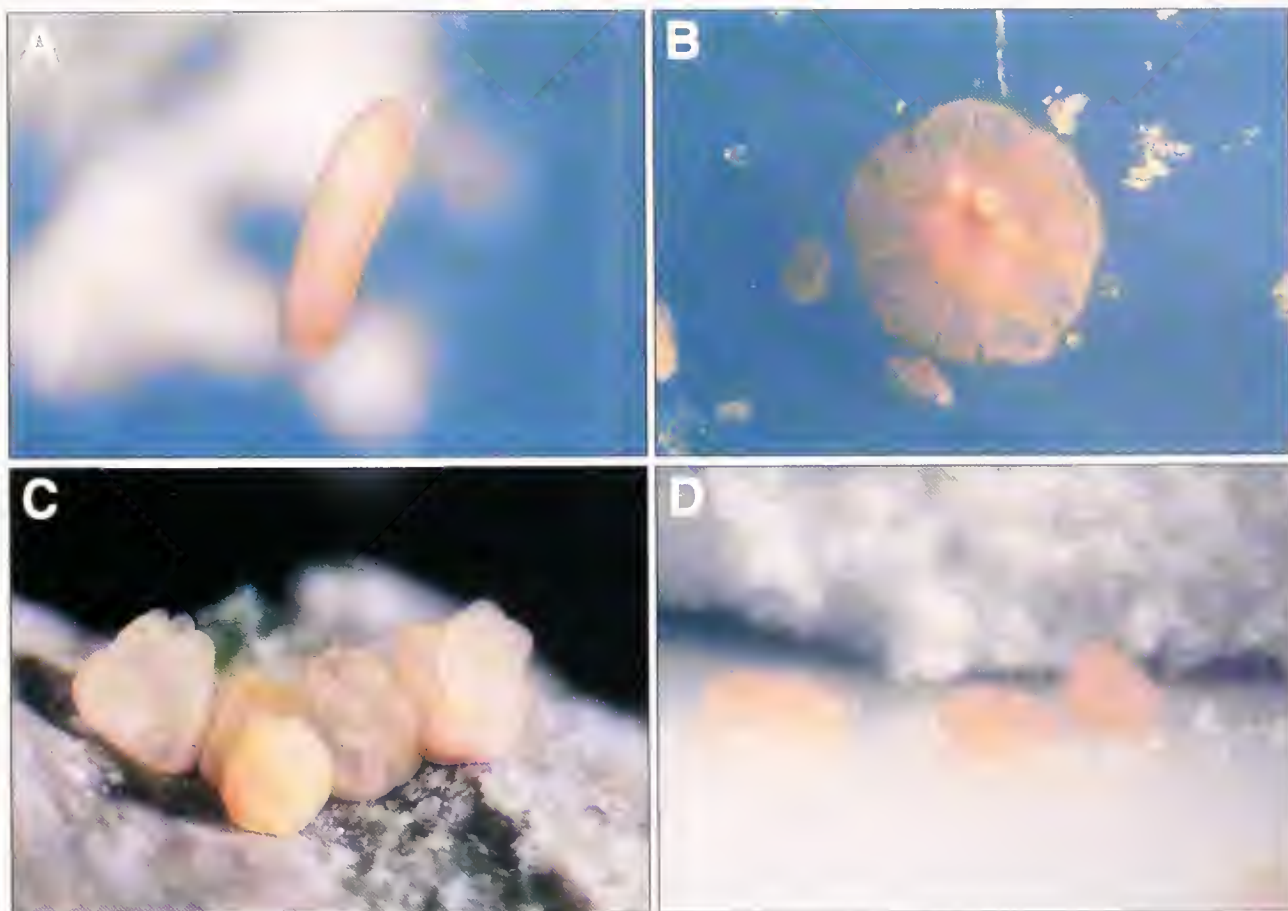


Figure 2. Larval behavior and early metamorphic changes of Pacific acroporid corals in response to a morphogenic cue associated with Pacific crustose red algae. (A) Typical shape of soft-bodied larva ≥ 3 days post-fertilization: *A. digitifera* larva, 8 days post-fertilization, swimming normally in the water column. (B) Final stage of metamorphosis; formation of radial skeletal elements (septa and costae) and elevation of central area around mouth: *A. nasuta* larva, 8 days post-fertilization, incubated with *Peyssonnelia* sp. (C) Early-stage metamorphosis (4 h) of a mixture of larvae of *A. nasuta*, *A. digitifera*, *A. tenuis*, *A. formosa*, *A. gemmifera*, *A. florida*, *A. sp. 5* on whole specimens of *H. reinboldii* and *Peyssonnelia* sp. (D) Metamorphosis of *A. florida* larvae in response to inductive molecules purified from *H. reinboldii* and coupled to resin beads. In (A) and (B) the assays were as described in Fig. 1. In (C) 20 competent larvae of each of seven species were incubated together in 200 ml FSW with or without small intact specimens of *Peyssonnelia* sp., *Hydrolithon reinboldii*, and *Lobophora variegata*. In (D) the resin with adsorbed inducer was the same preparation as that assayed in Table I; larvae were batchmates of those in (C).

sexual reproduction in which cross-fertilization occurs internally, and subsequent larval development and brooding occur within the maternal polyps.

The possibility of similar cue recognition in members of these two families was first suggested by the fact that the algal species (*Hydrolithon reinboldii* and *Peyssonnelia*) that are shown here to induce metamorphosis of Pacific acroporid larvae have congeners in the Caribbean that induce metamorphosis of agariciid larvae (5–10). This suggestion is confirmed by the demonstration (Table I) that the Pacific alga, *Hydrolithon reinboldii*, contains an extractable polymeric morphogen that is apparently in the same class of molecules as one previously identified in its Caribbean congener, *H. boergesenii*.

Fragments of both the Caribbean and Pacific algal congeners (*Hydrolithon* and *Peyssonnelia*) induced levels of metamorphosis in *Agaricia humilis* larvae that are not significantly different (Table I; one-way ANOVA: $F = 1.59$, $df = 4$, $P = 0.198$). Moreover, when the same procedures developed for biochemical purification, characterization, and coupling (7, 9, 15) of the polymeric inducer of agariciid larvae from *H. boergesenii* to a hydrophobic resin were applied to *H. reinboldii*, the purified molecule adsorbed to resin was recognized by *A. humilis* larvae (Table I). Larval settlement and metamorphosis of at least seven species in two genera of Caribbean agariciids is strictly dependent on chemosensory recognition of a unique class of sulfated glycosaminogly-

Table 1

Metamorphic response of larvae of the Caribbean coral, *Agaricia humilis*, to inducers from Caribbean and Pacific algal congeners

Inducer	n	Metamorphosis at 48 h (mean % $\pm$ std. error)
Caribbean		
None	10 ¹ + 30 ²	0 $\pm$ 0
<i>Hydrolithon boergesenii</i> fragments	25 ¹ + 30 ²	62 $\pm$ 9.1
<i>Peyssonnelia</i> sp. fragments	30 ²	73 $\pm$ 16.1
Control resin	10*	0 $\pm$ 0
<i>Hydrolithon boergesenii</i> inducer on resin	10*	90 $\pm$ 6.2
Pacific		
None	10 ¹ + 30 ²	0 $\pm$ 0
<i>Hydrolithon reinboldii</i> (form iii) fragments	30 ²	53 $\pm$ 8.4
<i>Hydrolithon reinboldii</i> (form iv) fragments	20 ¹ + 30 ²	56 $\pm$ 7.8
<i>Peyssonnelia</i> sp. fragments	30 ²	87 $\pm$ 6.7
Control resin	10 ¹	0 $\pm$ 0
<i>Hydrolithon reinboldii</i> inducer on resin	10 ¹	30 $\pm$ 6.2

Competent *A. humilis* larvae were incubated in 10 ml FSW with or without additions as shown: none = FSW alone; fragments = algae prepared as previously described (6, 7, 9); control resin = hydrophobic interaction chromatography (HIC) resin (9, 15) with no adsorbed chemical inducer; inducer on resin = HIC resin with adsorbed inducer purified from the indicated alga (9, 15). *n* = the total number of larvae tested in each condition in replicate tests (5 larvae/test) performed either in May 1995 (¹), May 1996 (²), or earlier (*, data from 9). Metamorphosis means development to the single-polyp stage (refs. 5, 15; and Fig. 2B). Percentages were arcsine transformed for statistical analyses. Brooded *A. humilis* larvae were obtained by spontaneous release from parental colonies in the laboratory rearing facility at UCSB, under conditions previously described (6, 7, 9); *H. boergesenii* and *Peyssonnelia* sp. were collected from Bonaire, Netherlands Antilles; *H. reinboldii* (forms iii & iv) and *Peyssonnelia* sp. came from Akajima, Japan. Fragments of all algae were prepared, shipped (-20°C), stored (-80°C); the inducers were purified from fragments and adsorbed to HIC resin by procedures developed with *H. boergesenii* (7, 9, 15). Resin assays: 20 mg resin with or without adsorbed inducer from *H. boergesenii* or from *H. reinboldii* (form iv).

can (7, 9) that is associated with the cell walls of a number of Caribbean crustose red algae (5, 7–9, 15). Enzymatic and biochemical analyses demonstrated that this inductive polymer has a molecular weight of 5–10 Kd and does not induce metamorphosis in coral larvae that are not induced by the intact parental algae (7). Both the biochemical characterization of the inducer isolated from an inductive Pacific alga and its recognition by *A. humilis* larvae indicate that this morphogen belongs to the same class of algal cell-wall polysaccharide as the compound obtained from Caribbean red algae. As we show here, acroporid larvae assayed at Akajima metamorphosed in response to both *H. reinboldii* fragments and intact algae (Figs. 1, 2B, C), as well as to this same

batch of resin-adsorbed morphogen purified from *H. reinboldii* (Fig. 2D); when incubated with resin lacking the adsorbed molecule, larvae of the Pacific corals remained arrested with no metamorphosis. Settlement and metamorphosis in the tested *Acropora* species are thus apparently induced by chemosensory recognition of the same class of algal sulfated glycosaminoglycan.

The acroporids and agariciids are members of the recently defined clade of complex scleractinians (16). In experiments otherwise identical to those above, larvae of the mass-spawning faviid corals from Akajima—*Favia fava*, *Goniastrea retiformis*, and *Cyphastrea* sp.—members of a taxonomically distinct clade of robust corals (2, 16, 17), also exhibited a strict requirement for the same class of algal morphogen recognized by the complex coral larvae. Thus, the inducer molecule purified from the two Pacific algae, *Hydrolithon reinboldii* and *Peyssonnelia* sp. (each adsorbed to separate batches of hydrophobic resin) induced metamorphosis of *Cyphastrea* sp. larvae. There was no spontaneous metamorphosis in the presence of resin with no adsorbed cue. Fragments of the intact alga *H. reinboldii* induced metamorphosis of *Cyphastrea* sp., whereas seawater alone, dead coral branches, and the brown alga *L. variegata* produced no metamorphic response. Similarly, *Favia fava* and *Goniastrea retiformis* metamorphosed in response to fragments of intact *Peyssonnelia* sp., but were unresponsive to *L. variegata* and seawater alone. Relatively few of these faviid larvae were available, which limited the testing of the other algae and purified inducers adsorbed to resin with each coral species. The results, however, demonstrate the stringency and specificity of the requirement of these robust corals for the same class of algal inducer (A. N. C. Morse *et al.*, University of California, Santa Barbara, in prep.).

The algal-cue-dependent settlement and metamorphosis of agariciids described here has been shown to operate effectively in the ocean as well as in the laboratory (9), and to contribute to substratum specificity of agariciid recruitment in the natural environment (6). We further suggest that this mechanism may enhance the probability of successful reproduction of coral species that depend on cross-fertilization. In mass-spawning corals the success of cross-fertilization of gametes in the plankton depends on a rapid encounter with gametes from another colony of the same species, and thus on the propinquity of reproductive colonies of the same species. Similarly, species with internal cross-fertilization depend on the transfer of sperm from one colony to another. Field manipulation of colony distances in *Agaricia humilis* reveals that colonies must be ≤ 2 m from their nearest neighbor for successful production of larvae (P. T. Raimondi and A. N. C. Morse, University of California, Santa Barbara, in prep.).

The evidence presented here indicates that representa-

tive species of three very large families of corals, the Acroporidae, Faviidae, and Agariciidae, have evolved similar morphogenic requirements and chemosensory signal recognition systems for larval recruitment from the plankton to the reef. This mechanism is independent of their geographic histories and of their modes of sexual reproduction. Our findings indicate that species in these three families evolved chemosensory receptors that recognize the same class of required chemical morphogen. Unless this mechanism arose independently multiple times, the results suggest an adaptation of a common ancestor. Our own findings, coupled with recent revisions of the evolutionary histories of scleractinian corals based on both molecular phylogenetic analyses (of both nuclear and mitochondrial genes) and morphometric and palaeontological studies (2, 16, 17, 18), suggest that this common mechanism is relatively old. It appears to predate not only the phylogenetic and geographic divergence of the corals we studied, but also the emergence, at 240 Ma, of the mineralized coral skeleton (16, 18).

Acknowledgments

We thank M. Hatta and T. Sugiyama for assistance with gamete fertilization, and M. H. Carr, L. A. Espada and A. Stewart-Oaten for assistance with statistical analyses. We thank A. Alldredge, J. Connell, E. DeLong, S. Gaines, A. Kuris, and D. Morse for helpful suggestions for the manuscript. The project was made possible by the interest and generous support of S. Hosaka, and by grants to A. M. from NSF, Division of Ocean Sciences, the NOAA National Undersea Research Program, and the Jean and Katsuma Dan Fellowship from the Marine Biological Laboratory, Woods Hole, Massachusetts.

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Activity of Lactate Dehydrogenase but Not Its Concentration of Messenger RNA Increases With Body Size in Barred Sand Bass, *Paralabrax nebulifer* (Teleostei)

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*In white skeletal muscle of conspecific pelagic fishes, the activities of enzymes associated with anaerobic glycolysis, e.g., lactate dehydrogenase (LDH), usually scale positively with increasing body size; this pattern is opposite to that found for enzymes of aerobic metabolism, which decrease in mass-specific activity with size (1–3). The higher mass-specific capacities for anaerobic ATP generation in larger conspecifics are thought to facilitate conservation of high-speed (“burst”) swimming ability in fishes of different sizes (1). To investigate the mechanisms responsible for scaling of LDH activity in white muscle, we quantified LDH activity, total RNA, and the specific mRNA for LDH-A (the skeletal muscle isoform of the enzyme) in white muscle of *Paralabrax nebulifer*, the barred sand bass. We also measured total protein concentration and the concentration of actin, the major protein of thin filaments, and its specific mRNA. Although LDH activity scaled significantly with body size as predicted (1–4), no other biochemical trait measured showed a significant size-dependent concentration. We conclude that the regulation of LDH activity in white muscle of this species is not governed by LDH-A mRNA concentrations, but rather by one or more other mechanisms, for example, an elevated rate of translation of LDH message or a reduced rate of degradation of LDH-A in larger fish.*

Among pelagic fishes with the ability to swim in strong bursts, conspecifics show large size-dependent changes in muscle enzymatic activity (1–3, 5). When the activities of glycolytic enzymes like LDH and pyruvate kinase, which are critical for ATP generation under oxygen-limiting conditions, are normalized on the basis of units per gram of muscle, they show an increase in larger conspecifics. In contrast, enzymes associated with aerobic respiration, e.g., citrate synthase and cytochrome *c* oxidase, decrease in accord with well-known concepts for scaling of whole-organism aerobic respiration (1–3, 6). The positive scaling of glycolytic enzymatic activities with body size has been interpreted as providing size-independent capacities for high-speed burst swimming, i.e., for sustaining an ability to maintain relative swimming speeds (body lengths s^{-1}) that are identical in different sized individuals of a pelagic species with subcarangiform locomotion (1).

The mechanisms that regulate enzyme concentrations in size-specific patterns are unknown. Clearly, the opposite scaling patterns noted for enzymes of anaerobic versus aerobic pathways of ATP generation indicate that regulation of overall protein synthesis or degradation is not a sufficiently specific mechanism to bring about this type of regulation.

To address this issue, we examined size-related biochemical properties of white skeletal muscle of the barred sand bass, *Paralabrax nebulifer*, an active subcarangiform swimmer. Fish were collected by seining on beaches in San Diego, California, and maintained in flowing, ambient seawater at the Scripps Institution of

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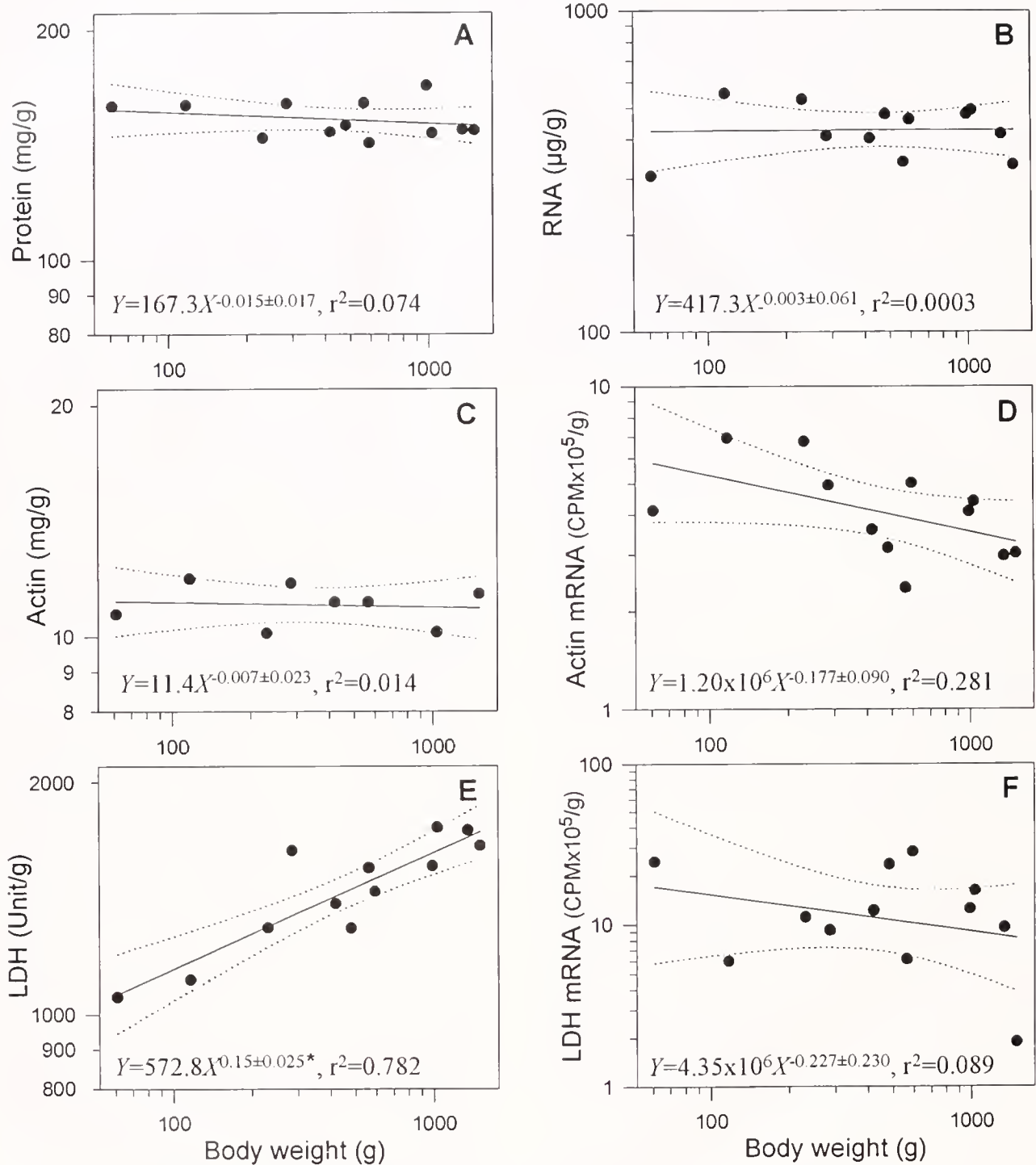


Figure 1. The relationships between body mass and concentrations or activities of proteins and RNAs in the white epaxial muscle of the barred sand bass, *Paralabrax nebulifer*. All the biochemical parameters are expressed as values per gram wet weight, and each dot represents the mean value for duplicate measurements. The equation used is of the general form $Y = aX^b$. Where the slope (b) of the regression line is significantly different from 0 ($P < 0.05$), an asterisk (*) is shown. (A) Muscle total protein concentration as a function of body weight. The total muscle homogenate was used in the bicinchoninic acid assay (13) for determining protein concentration. (B) Muscle total RNA as a function of body weight. The total RNA was determined by measuring a small aliquot of total muscle RNA extract (7) at OD 260. (C) Muscle actin

Oceanography. They were fed *ad libitum* on chopped squid every other day for 3 weeks before sacrifice (by cervical transection). Specimen weights ($n = 12$) ranged from 61 g to 1508 g (Fig. 1). Immediately after sacrifice, two pieces (about 2 g each) of white skeletal muscle were removed from the epaxial white musculature near the base of the first few dorsal spines of the fish. Each tissue sample was quickly minced on a glass plate held at ice temperature and then extracted for RNA, following the protocol of Yang and Somero (7). The rest of the specimen was immediately frozen at -70°C , for later use in measurements of total protein and actin concentration and LDH activity. LDH was chosen as an enzyme with a strong positive scaling pattern (1–4), and actin was selected because its concentration typically varies little within or among fish species (8). The levels of mRNAs for LDH-A and actin were determined as described by Yang and Somero (7). A specific probe for the A-isoform of LDH was used because this is the only isoform of LDH found in white muscle of this species (Linda Z. Holland, Scripps Institution of Oceanography, pers. comm.). A probe for the alpha-isoform of actin was generously provided by Dr. J. Eldridge (National Institutes of Health).

Figure 1 shows the relationship between body mass and each of the biochemical properties measured. Only LDH activity showed a significant dependence on body size, a result in accord with previous studies of many pelagic fishes, including a congener, *Paralabrax clathratus* (1, 3). In both congeners, actin concentration did not scale with body size. However, in *P. clathratus* total protein did scale positively, albeit weakly ($R^2 = 0.36$), with body size, whereas no significant trend was found in *P. nebulifer* (Fig. 1).

The lack of size-related variation in the concentrations of LDH mRNA and actin mRNA suggests that the different scaling patterns for these two proteins are not a simple consequence of different steady-state concentrations of their specific messenger RNAs. Were mRNA levels the primary determinant of protein concentration, then the level of LDH mRNA would rise with increasing body size, in parallel with the observed increase in LDH activity. The lack of correlation between LDH activity

and LDH mRNA concentration in muscle of *P. nebulifer* agrees with the results from studies of different tissues of rats (9–10); those studies also failed to show any significant correlation between the two variables. In these particular cases it appears that LDH activity may be regulated by post-transcriptional events. In contrast to these findings, Crawford and Powers (11) reported a strong correlation between the activity of LDH-B and levels of LDH-B mRNA in populations of the fish *Fundulus heteroclitus* from the northern and southern extremes of its distribution range. They hypothesized that the higher enzymatic activities and mRNA levels present in the northern, more cold-adapted population could have resulted from variations in *ldh-b* gene regulation. The mechanisms for regulating enzymatic activity in response to differences in temperature may differ from those that establish the size-dependent patterns in enzyme levels noted in this and earlier studies (1–4). To elucidate the mechanism or mechanisms responsible for size-related variation in LDH activity, as well as for other enzymes that exhibit scaling, it will be necessary to quantify the rates of synthesis and degradation of these enzymes and their mRNAs.

The observation that the total RNA concentration in white muscle of *P. nebulifer* is independent of size was surprising in view of the finding that rates of protein synthesis in fish muscle may change with size and age (12). The RNA pool in a cell is composed primarily of ribosomal and transfer RNAs. Thus, total RNA concentration provides an index of the amount of protein synthetic machinery present in the cell. If the size-independence of total RNA is characteristic of fish white muscle, then size- or age-specific rates of protein synthesis may be governed by factors other than the concentration of the ribosomal protein synthetic machinery present in the cell.

Acknowledgments

The authors thank Mr. Ronald R. McConnaughey for his assistance in collecting fish and Dr. J. Eldridge for sharing the actin cDNA. We also thank Ms. Linda Z. Holland and Dr. Douglas Bartlett for their help and sug-

concentration as a function of body weight. The actin concentration was measured by the DNase I inhibition assay (8). (D) Relative concentration of muscle actin mRNA as a function of body weight. To determine the relative actin mRNA level, an anti-sense RNA probe derived from chicken α -actin was used for the liquid hybridization assay (7). (E) Activity of muscle LDH as a function of body weight. White muscle was homogenized with 10 volumes of 10 mM Tris/Cl, pH 7.2 (20°C). LDH activity was assayed by adding 20 μl of this homogenate to 2 ml of medium containing 80 mM imidazole/Cl buffer (pH 7.0, 20°C), 100 mM KCl, 200 μM NADH, and 5 mM pyruvate. (F) Relative concentration of muscle LDH mRNA as a function of body weight. The anti-sense RNA probe derived from *Sebastes albus* LDH-A₄ cDNA was used in liquid hybridization assays (7).

gestions on RNA hybridization assays. The research was supported by National Science Foundation grant IBN92-06660.

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Acute Cytotoxic Allogeneic Histoincompatibility Reactions Involving Gray Cells in the Marine Sponge, *Callyspongia diffusa*

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Abstract. A variety of procedures were used in a study of the histoincompatibility reactions of *Callyspongia diffusa*. Rejection reactions as traditionally tested between laterally apposed intact fingers cut from two different sponges require about a week of contact to exhibit cytotoxicity. In a miniaturized assay involving reactions between small pieces of tissue snipped from sponges with scissors and pushed together on an insect pin, cytotoxicity is evident within 48 hours of contact. Reactions of cells dissociated by divalent cation removal and allowed to reaggregate in seawater were also studied. Aggregates produced from allogeneic mixtures of cells from two individuals were killed by internal cytotoxic reactions within 36 hours of the initiation of aggregation. After only one hour of aggregation, aggregates from allogeneic mixtures were significantly smaller than aggregates of cells from a single individual. This rapid slowing of aggregation is the earliest response to allogeneic contact that we noted and does not appear to reflect early cytotoxic processes. Apposition of an aggregate containing cells from one sponge to an aggregate containing cells from a second individual leads to mutual destruction. Aggregates harvested and apposed 4 hours after initiation of aggregation begin to show mutual cytotoxicity at 36 hours of contact. Aggregates placed in contact 48 hours after the initiation of aggregation exhibit cytotoxicity within 8 hours. These rapidly reacting 48-hour aggregates exhibit a pronounced accumulation of gray cells at the boundary of allogeneic contact by 8 hours. These results are interpreted as indicating at least five steps in the histoincompatibility reactions of *C. diffusa*: (1) recognition soon after allogeneic contact; (2) genera-

tion of signals that suppress cell aggregation and cell movement and attract gray cells to the boundary of contact; (3) acceleration of the sponge immune response—including the responsiveness of gray cells to accumulate at the boundary of allogeneic contact—by tissue trauma produced when the tissue is cut or dissociated it into individual cells; (4) arrival of gray cells at the boundary of allogeneic contact; and (5) initiation of cytotoxic processes.

Introduction

The invertebrate immune system must protect the individual animal from a full range of parasitic and pathogenic threats (Ratcliffe *et al.*, 1985). For sessile species, individual-specific recognition may also prevent cellular parasitism between contacting individuals of the same species (Buss, 1987). Very specific, immune-like histoincompatibility reactions occur in every invertebrate phylum (Cooper *et al.*, 1992) including sponges, the simplest metazoans (Hildemann *et al.*, 1979). Because these reactions are usually very slow, and not well defined histologically, cells functioning like vertebrate T or B cells are not evident in any invertebrate. The idea that evolutionary precedents for specific recognition events characteristic of these vertebrate immune cells cannot be found in invertebrates is often expressed (Klein, 1989; Janeway, 1992; Smith and Davidson, 1992).

This laboratory has observed very rapid allogeneic recognition reactions involving the intimate participation of a specific cell type—gray cells—in the marine sponge *Microciona prolifera* (Humphreys, 1994). The rapidity and selectivity of these reactions have led us to focus again on the possibility of specific cellular recognition in the immune reactions of invertebrates (Humphreys and

Reinherz, 1994). Following Burnet's (1971) original suggestion, we have emphasized direct, specific, self recognition as the guiding principle for invertebrate immunity. Conceptually, if invertebrate immunocytes recognize individual self histocompatibility marker proteins, they can immediately, upon contact, recognize as non-self all cells that do not display the identical self-marker proteins. The ability of invertebrate immunocytes to recognize self could be likened to the ability of vertebrate T-cells to recognize self major histocompatibility complex (MHC) proteins. Just as vertebrate T-cells learn to recognize self MHC proteins through the process of positive selection, one can imagine that during differentiation, invertebrate immunocytes learn to recognize self proteins encoded by the specific alleles that the individual animal has inherited at a polymorphic locus (or loci) that encodes self marker proteins (Humphreys and Reinherz, 1994).

The tropical marine sponge *Callyspongia diffusa* was used in some of the first experiments on histoincompatibility in sponges (Hildemann *et al.*, 1979). Subsequently, graft rejection reactions have been described in a variety of sponge species and two distinct rejection modalities occur (Van de Vyver and Buscema, 1988). Some sponges—for example, *C. diffusa*—produce a nonspecific cytotoxic reaction that kills all cells within a few millimeters of the boundary of foreign contact (Hildemann *et al.*, 1980). Other species, such as *M. prolifera*, carry out an encapsulation-like process and produce a barrier, often containing collagen, between the contacting tissues (Humphreys, 1994). Our observations on *M. prolifera* suggest immediate cellular recognition upon allogeneic contact with the release of a chemokine that attracts the gray cells to accumulate in the zone of contact and organize the formation of the collagen layer (Humphreys, 1994). Although reactions of other species of sponges have been examined in considerable histological detail (Van de Vyver and Buscema, 1988; Smith and Hildemann, 1986a, b), few other clues concerning the cellular and mechanistic bases of recognition or rejection reactions have emerged.

Encouraged by a report that mixed aggregates of dissociated cells from two individuals of *C. diffusa* undergo a rapid rejection reaction which causes self annihilation of the aggregates within two days (Johnston, 1988), we began to explore the possibility of more rapid assays of recognition in this species. In fact, we describe cellular assays that can detect recognition reactions within an hour of allogeneic contact. We have also discovered acceleration and maturation processes that are components of the reacting immune system. In addition we have described gray cells in *C. diffusa* and have shown that recognition signals cause them to accumulate at the zone of allogeneic contact in association with the cytotoxic process. These results provide a beginning for pro-

ductive cellular level analysis of allogeneic recognition in this species.

Materials and Methods

Sponges

The brilliant purple sponge *Callyspongia diffusa* (Ridley) was collected from the shallow reefs around Coconut Island, Kaneohe Bay, Oahu, Hawaii, and maintained in the running seawater tanks at the Kewalo Marine Laboratory. Sponges of 10 to 50 grams or more survive and continue to grow slowly for six months to one year in these tanks in spite of the excision, from time to time, of small portions of their tissue for experiments.

Histocompatibility assays

In addition to the original grafting system described for *C. diffusa* (Hildemann *et al.*, 1979) in which two intact fingers, typically several centimeters long, are tied together on a 2" × 3" microscope slide maintained in seawater, we have developed a miniaturized assay. Two small pieces of tissue a few mm in dimensions, snipped from the sponge with scissors, are pushed together on the end of a #0 stainless steel insect pin that is incubated standing in 3 ml of filtered seawater in a well of a 24-well tissue culture plate (Falcon type 3047) shaking at 50 rpm on a rotatory shaker. The seawater is changed daily.

We also assayed the histoincompatibility reactions that occur within aggregates of dissociated cells produced with mixtures of cells from two different sponges, as well as the reactions that occur between fully developed aggregates apposed in allogeneic pairs. The details of these assays follow a description of cell dissociation and reaggregation.

Cell dissociation and reaggregation

Sponge tissue (5 to 10 mm of a finger of the sponge, about 0.5 gram), is washed four times, 10 minutes per wash, with 10 ml per gram of tissue of calcium and magnesium free seawater (CMF-SW) (Humphreys, 1963) at room temperature, 22–25°C (lowering the temperature to 4°C kills *C. diffusa* cells). The washed tissue is then squeezed repeatedly in a bag of 100 μ m Nitex mesh to disperse the cells from the matrix into 10 ml CMF-SW per gram of tissue. The cell concentration of the suspension typically ranges from 10 to 20 × 10⁶ cells per ml. The viability of the cells is checked by dye exclusion, either with 1% trypan blue in CMF-SW or the live/dead viability/cytotoxicity kit of Molecular Probes, Inc. (L-3224). Freshly dissociated cells routinely have a viability greater than 95%. The dissociated cells are centrifuged at 1500 rpm for 1½ min at 15°C (Sorvall Rt6000B Refrigerated Centrifuge), resuspended at a concentration of 10 × 10⁶ cells/ml in 0.45 μ m Millipore filtered SW supple-

mented with 0.01% bovine serum albumen (BSA-SW; the addition of the very dilute BSA to the SW seems to improve the viability and health of the cells and aggregates). To produce aggregates, the cell suspension is immediately dispensed in 0.4-ml aliquots into 16-mm diameter wells of 24-well tissue culture plates (Falcon type 3047). Aggregation is initiated by placing the multiwell plates on a rotatory platform shaker with a 3" diameter of rotation (Henkart and Humphreys, 1970) shaking at 50 rpm. The BSA-SW is gently replaced at 8 hours and then every day. For time series determinations, replicate multiwell plates are started to provide an undisturbed sample for each time point determination. Aggregates are photographed with direct illumination under a Wild M5 binocular microscope using Kodak Ektachrome film.

Histoincompatibility reactions in aggregates

To examine the reactions of allogeneic mixtures of cells, aggregates containing cells from two different sponges are produced. Two 0.2-ml aliquots of dissociated cell suspension from each of two sponges are mixed in the same aggregation well of a multiwell plate, which is then placed on the rotatory shaker.

The reactions of pairs of aggregates are examined by manually apposing two aggregates, each made up of cells of only one sponge. Aggregates are selected individually from the aggregation wells using a micropipette with an 0.5 mm bore under a binocular microscope. Aggregates 0.2 to 0.3 mm in diameter are selected and paired in the bottom of a well of a 96-well, U-bottom Micro Test III flexible Assay Plate (Falcon type 3911) with 0.1 ml of filtered seawater. For allogeneic reactions, an aggregate from one aggregation well containing cells of only one sponge is apposed to an aggregate from a different aggregation well containing only cells from a different sponge.

Gray cell fractionation

Gray cells are purified by centrifuging a preparation of dissociated cells in CMF-SW layered over a cushion of 8% Ficoll, 16% sodium diatrizoate in CMF-SW at 3000 rpm in an IEC refrigerated centrifuge at 15°C for 15 minutes. The pellet is resuspended in CMF-SW and consists of about 50–70% gray cells (See Fig. 6A and 6B).

Results

Tissue grafting

The original histocompatibility experiments with *C. diffusa* were replicated by tying two fingers cut from different sponge individuals side by side on a glass slide maintained in running seawater (Hildemann *et al.*, 1979). As described previously, the fingers adhere strongly for several days without discernible differences

between autogeneic and allogeneic contacts. At about 6 days, depending somewhat on temperature (Johnston *et al.*, 1981), the organization of the cells along the zone of contact changes visibly, and 24 to 36 hours later cellular degeneration becomes apparent along the boundary of contact. As already analyzed extensively (Jokieli *et al.*, 1982), certain allogeneic combinations of specific individuals reproducibly react weakly or slowly, presumably due to genetic similarity of individual sponges in these populations. For the results presented here, reactive sponge pairs were used in all experiments.

We miniaturized the histocompatibility assay by examining the reactions of two small pieces of tissue pressed together on an insect pin. These pieces adhere rapidly in both autogeneic and allogeneic combinations (Fig. 1). The former heal together and remain healthy indefinitely. The latter usually exhibit degeneration along the zone of contact beginning by 48 hours. Figure 1 shows insect pin assays of the three autogeneic and the three allogeneic pairings possible between three individual sponges after 72 hours of contact, when degeneration of tissue is very evident along the boundary of contact in the allogeneic pairs. An interesting feature of the reacting tissue in these insect pin assays, evident on pins C and D in Figure 1, is the migration of cells onto the pin away from the area of cytotoxic reactions. Such emigration is not observed in non-reacting tissue. All living cells are usually eliminated from the original sponge matrix in these miniaturized grafts by 4 days. Under this protocol—involving both wounding of the contact surfaces and trauma to the sponge tissue by the crushing action of the scissors during the process of cutting the small pieces—allogeneic reactions are significantly more rapid with cell degeneration evident by 48 hours. This may be contrasted with reactions in about 7 days for laterally apposed complete fingers that have not been traumatized by cutting in the zone of contacting tissue (Hildemann *et al.*, 1980).

Cell reaggregation

Following the observations of Johnston (1988) on *C. diffusa*, we examined histoincompatibility reactions of allogeneic mixtures of reaggregated cells. We confirmed that cells, mechanically dissociated in seawater (SW) and allowed to settle on glass or plastic, aggregate and differentiate into small functional sponges within a few days when they are derived from one individual. In contrast, allogeneic mixtures of cells from two sponges die within 48 hours. We also examined these reactions with cells that had been dissociated by the removal of divalent cations (Humphreys, 1963), returned to complete SW, and reaggregated in suspension in wells of a multiwell plate on a rotatory shaker. Similar killing of mixed aggregates occurs under these conditions.

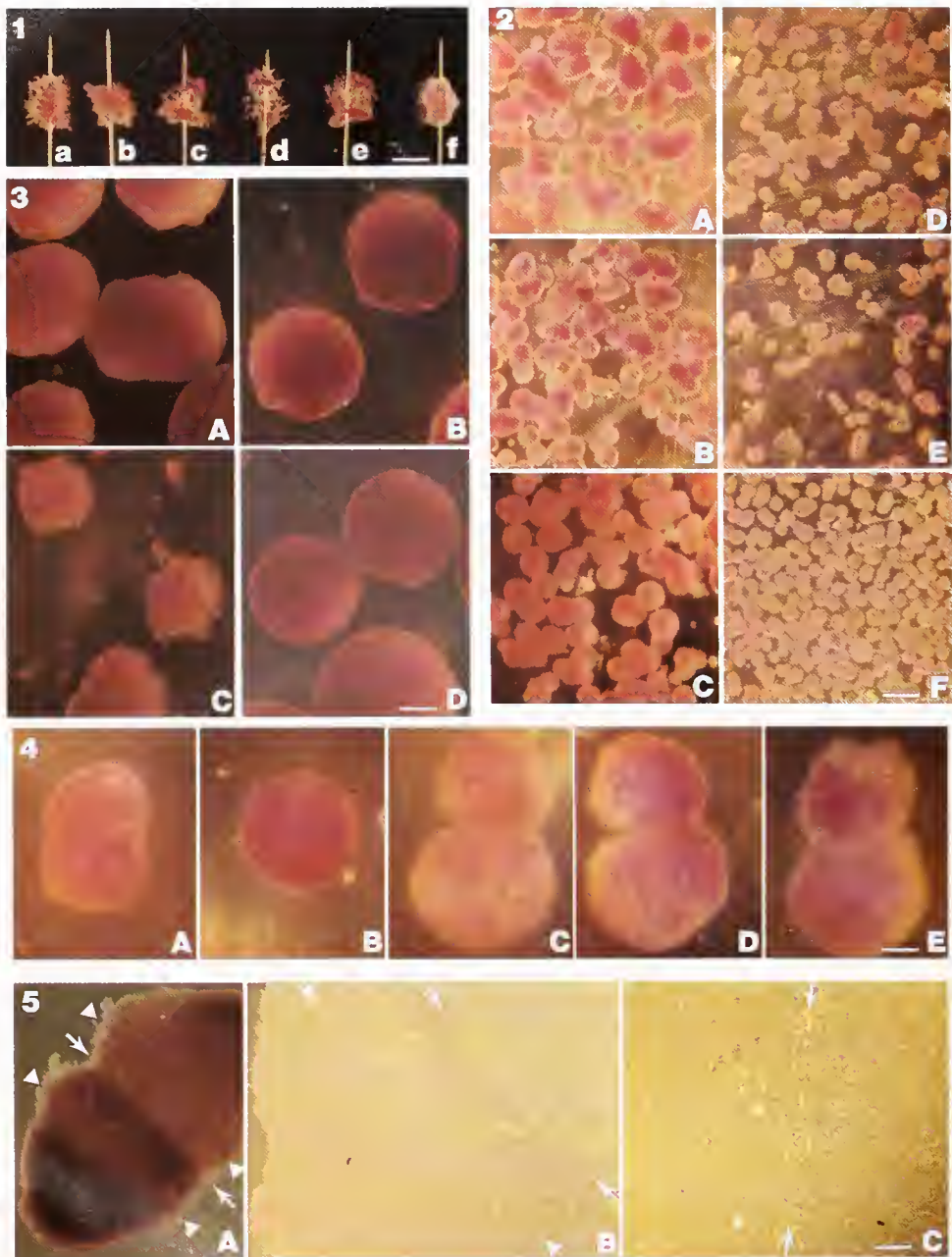


Figure 1. Histoincompatibility assay. The three autograft and the three allograft combinations possible between three individual sponges are shown 72 hours after apposition of pieces of tissue on insect pins. Two apposed pieces of tissue from the same sponge remain healthy and heal together (a, b, f). Two pieces of tissue from different sponge individuals react and create a zone of cell death exposing the skeletal matrix along the zone of allogeneic contact (c, d, e). Bar equals 0.4 cm.

Figure 2. Aggregates from dissociated cells from each of three individual sponges. Aggregates of pure populations of cells from the three sponges (A, B, C), and the three possible pair-wise allogeneic mixtures of dissociated cells from the three sponges (D, E, F), 2 hours after the initiation of aggregation in suspension shaker cultures. The aggregates of mixed cells from two individuals in D, E, and F are significantly smaller than aggregates from individual pure cell aggregates in A, B and C. Bar equals 0.5 mm.

Figure 3. Cytotoxic histoincompatibility reactions in aggregates containing an allogeneic mixture of dissociated cells derived from two different sponges. A. A lumpy surface at 20 hours of aggregation is the first evidence of cytotoxic reactions in mixed aggregates. B. By 21 hours the roughened appearance of the aggregate surface has become more pronounced. C. By 26 hours the mixed aggregates have begun to fall apart into smaller cell clumps which soon disintegrate completely. D. Aggregates of cells derived from one individual remain smoothly spherical as shown after 82 hours of aggregation. Bar equals 0.2 mm.

Table I

Average diameter of the ten largest aggregates in shaker wells after 2 hours of aggregation

Cells from sponge	Aggregate size (mm)
T	0.42 ± 0.07
W	0.40 ± 0.07
X	0.34 ± 0.05
T + W	0.24 ± 0.03
T + X	0.23 ± 0.02
W + X	0.23 ± 0.02

Suspensions of dissociated cells from an individual sponge adhere rapidly, forming ragged clumps within minutes. These aggregates in suspension become smooth and rounded (Fig. 2A, B, C) and attain maximum dimensions of 0.3 to 0.5 mm (Table I) by 2 hours. Although further aggregation after 2 hours is limited by the shearing forces in the shaker wells, these aggregates remain very adhesive and will adhere to each other or to the substrate within minutes if shaking is discontinued. When two spherical aggregates adhere but remain in suspension and shaking is reinitiated, they rapidly round up into a single larger spherical aggregate. If aggregates are allowed to attach to the substrate, they flatten and differentiate into functional sponges in 48 to 72 hours. Aggregates of cells from a single individual remain viable and healthy in suspension for days (Fig. 3D).

Allogeneic mixtures of cells in SW on a rotatory shaker also adhere rapidly, but aggregation slows appreciably in the first hour. By two hours (Fig. 2D, E, F), when they have attained maximum diameter, they are 0.20 to 0.25 mm (Table I), which is about 0.6 the diameter and one fifth the cell number of the aggregates formed from unmixed cells. The slowing of aggregation is not due to obvious cytotoxic processes. The viability of the cells, examined by dye exclusion after dissociating the aggregates with EGTA, remained over 95% for up to 20 hours. If shaking is stopped, these allogeneic aggregates adhere to each other but do not tend to round up into a single

sphere. Instead, they maintain their original aggregate identity within the clump. These aggregates, when allowed to attach to the substrate, never flatten and differentiate. This rapid inhibition of cell aggregation and remodeling of aggregates provides an interesting early indication that allogeneic recognition has occurred.

At about 24 hours, cell degeneration becomes manifest in the allogeneic mixed aggregates maintained in suspension. The first indication of change is a lumpy appearance of the previously smooth outer surface of the aggregates (compare Fig. 3A with Fig. 3D). In the following 1–2 hours, the surfaces of the aggregates become rough and irregular (Fig. 3B). The aggregates soon begin to break open and fall apart into smaller clumps as shown at 26 hours (Fig. 3C). During this latter period the percentage of cells failing to exclude dye also begins to rise. Over the next few hours, as the remaining clumps of cells fall apart, the cells lyse.

Aggregate pairs

The ability of fully formed aggregates of dissociated cells from one sponge to carry out a histocompatibility reaction when contacted by an aggregate of cells from another sponge (Humphreys, 1994) was examined in *C. diffusa* by apposing round aggregates formed in suspension. When two aggregates of cells from the same individual are pushed together in SW in a conical well of a microtiter plate, they adhere within minutes, undergo substantial healing together by 2 hours (Fig. 4A), and form a single round aggregate by 4–6 hours (Fig. 4B). This remodeling of the aggregates from two spheres into one sphere reflects the tendency of rapidly migrating, adhesive cells to maximize adhesions as they move rapidly over each other (Steinberg, 1963).

If an aggregate of cells from one individual sponge is placed in allogeneic contact with an aggregate of cells from a different sponge, they also adhere within minutes but, as shown at 2 hours in Figure 4C, do not then lose their separate aggregate identities. Even though they continue to adhere tightly, they remain as two distinct

Figure 4. Allogeneic histoincompatibility reactions between apposed 24-hour aggregates of dissociated cells. A and B. Pairs of aggregates from the same sponge heal together rapidly as shown after two hours in A. By six hours in B the original two spherical aggregates have melded together to form one single, spherical aggregate. C, D, and E. Apposed aggregates from two different sponges adhere, but do not heal together, after 2, 6, or 12 hours of contact (C, D, and E, respectively). By 12 hours of contact, the aggregates have become lumpy, evidencing the initiation of cytotoxic reactions. Bar equals 0.1 mm.

Figure 5. Migration of gray cells to the zone of allogeneic contact in apposed 48-hour aggregates from two individuals. A. After 6 hours of contact a zone of less pigmented, light refractive tissue (arrowheads) parallel to the boundary of allogeneic contact (arrows) is evident in an aggregate pair viewed and photographed with incident illumination under a binocular microscope. B. The aggregate shown in A, which has been compressed under a coverslip and viewed and photographed at low power with DIC optics. The light zone can be seen to represent the accumulation of refractive cells which, at this magnification, appear only as the brighter objects scattered among the otherwise purplish tissue. C. The refractive cells ultimately congregate at the boundary of allogeneic contact. Bar equals 0.1 mm.

groupings of cells as shown after 6 or 12 hours in Figure 4D and E. In Figure 4E the edges of the aggregates near the boundary of allogeneic contact have become rough. Over the next 6 to 8 hours the aggregates will break down into smaller clumps of cells that proceed to disintegrate completely. The failure of the cells within the two adherent aggregates to round up into one spherical aggregate suggests that cell movement has been suppressed by the histoincompatibility reactions.

We also discovered that mature aggregates harvested 48 hours after initiation of reaggregation react more quickly than newly formed aggregates. Aggregates collected 4 to 6 hours after the start of aggregation exhibit cytotoxic processes only after 36 hours of contact. Aggregates 24 hours old react more quickly (Fig. 4) and 48-hour aggregates initiate cytotoxic processes within 8 hours of apposition. Thus, the processes responsible for the cell-killing reactions mature and proceed more rapidly in the older aggregates.

Gray cells

Because gray cells are intimately involved in the histoincompatibility reactions of *M. prolifera* (Humphreys, 1994), we examined the gray cells of *C. diffusa*. Previous histological studies on *C. diffusa* (Smith and Hildemann, 1986a, b) failed to identify gray cells. However, an examination of CMF-SW-dissociated, living *C. diffusa* cells under Kohler or differential interference contrast (DIC) optics revealed a small but distinct population of large, multigranular cells resembling gray cells. These cells,

concentrated by purification on a density gradient, are shown in Figure 6 A and B as photographed at high power with DIC optics. The characteristic feature of gray cells is the cytoplasm filled with densely packed, highly refractive, oblate granules. The gray cells do not contain the purple pigment evident in Figure 6A and B in the variety of cell types contaminating the gray cell preparation.

Gray cells were originally described in *M. prolifera* as appearing gray when viewed live under Kohler bright field microscopy (Wilson and Penney, 1930). Gray cells appear gray under Kohler optics because they are unpigmented, and their cytoplasm is completely filled with small granules of high refractive index that create an aura of darkness about the cell. Because the original designation of the gray cells in *M. prolifera* was based on their microscopic appearance, we refer to these corresponding cells in *C. diffusa* as gray cells. Gray cells represent about 1 to 3% of the total cells released from *C. diffusa* tissue by dissociation in CMF-SW.

In *M. prolifera*, gray cells accumulate at the boundary of allogeneic contact, creating a visible band in the reacting tissue (Humphreys, 1994). We examined reacting intact fingers, reacting pieces of tissue on insect pins, and reacting newly formed aggregates of *C. diffusa* to ascertain whether similar accumulations of gray cells occur at the boundary between reacting tissue in this species. Although there were suggestions in many experiments that gray cells were accumulating, we were unable to devise protocols that would yield striking and consistent accumulation of gray cells at the boundary of reaction

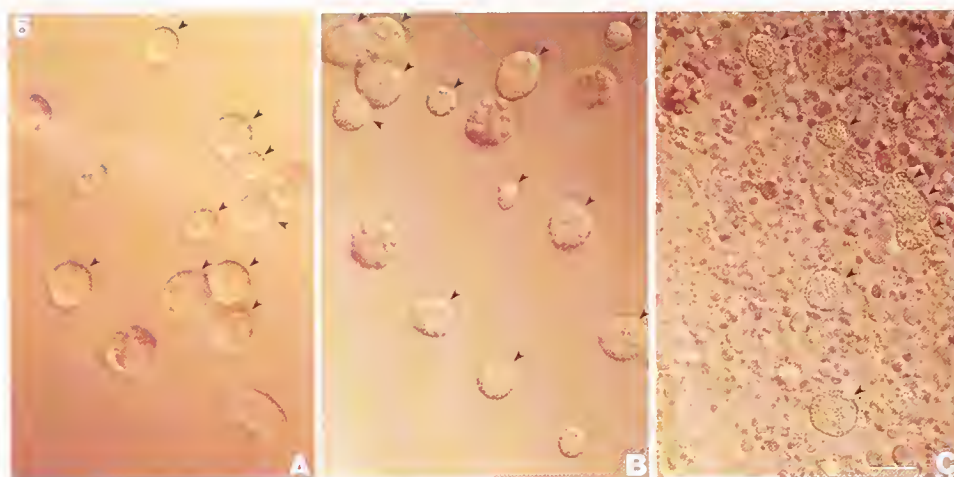


Figure 6. Gray Cells. A and B. Dissociated gray cells enriched by density gradient centrifugation C. Gray cells in a migrating band scattered among the purplish cells of a reacting 48-hour aggregate; 6 hours after the aggregates were apposed, they were compressed to 10 to 20 μm under a coverslip, examined, and photographed. Gray cells (marked by arrowheads) are bright, non-pigmented cells whose cytoplasm is packed with small oblate granules. The various non-gray cell types in the preparations can be seen to exhibit a diversity in number and type of cytoplasmic inclusions and the purple pigmentation characteristic of *C. diffusa*. Observed at 100 $\times$ with differential interference contrast optics. Bar equals 10 μm .

in these experimental settings. However, when 48-hour aggregates, which react in 8 hours, are apposed, a band of lighter colored tissue parallel to the boundary of contact became detectable within four to eight hours. A pair of reacting 48-hour aggregates, photographed 6 hours after pairing, exhibits such a band, and it seems to be converging on the boundary of allogeneic contact (Fig. 5A). If the adhering aggregates are greatly compressed under a cover slip until they are amenable to examination by DIC optics, an accumulation of gray cells is evident. A general view, taken with a 20 $\times$ DIC objective, shows the aggregate pair from Figure 5A compressed to one fifth its original thickness (Fig. 5B). The brighter spots arrayed parallel to the boundary of allogeneic contact among the otherwise lightly purple cells represent gray cells. If the aggregates are compressed even more and observed with a 100 $\times$ oil DIC objective, these brighter spots can be seen to be definitive gray cells with their densely packed oblate granules (Fig. 6C). One or two hours later, when the aggregates first exhibit signs of cytotoxic reactions, the gray cells are concentrated at the boundary of contact between the allogeneic cells as shown at low power in Figure 5C. We were unable to continue to find and identify the gray cells in the aggregates beyond this period during which the cytotoxic reactions begin. No accumulation of gray cells occurs in remodeling pairs of contacting aggregates of cells from the same sponge.

Discussion

We have studied the histoincompatibility reactions in the marine sponge *C. diffusa* and have developed novel modifications of this interesting experimental system that will help to elucidate the cellular and molecular mechanisms of immune recognition in the invertebrates. The histoincompatibility reactions in this species have been well studied (Hildemann *et al.*, 1979). Cytotoxic reactions between parabiosed intact fingers of two different sponges occur after a week and destroy a few millimeters of tissue along the boundary of allogeneic contact. These reactions have been followed by extensive histological examination (Smith and Hildemann 1986a, b; 1988) without a clear delineation of any specifically immune cells. The observations of Johnston (1988), that allogeneic mixtures of dissociated cells begin cytotoxic reactions within 48 hours, suggested to us that further examination of these more rapid reactions of dissociated cells might yield further insight into possible cellular mechanisms.

Our experiments present two new results that may be important for understanding the cell biology of recognition. We discovered an experimentally observable parameter, the rate of cell movement and aggregation, that changes within one hour of actual allogeneic contact, possibly reflecting the generation of a non-self recogni-

tion signal. This regulation of cell behavior can serve as a time- and possibly cell-proximal assay for the activation of the rejection reaction. It could prove useful for identifying the cells involved in recognition and signaling and for screening compounds, such as sugars, cyclosporin A, or monoclonal antibodies, which impinge directly on the early cellular events of recognition.

We have also established that *C. diffusa* has a population of gray cells that is specifically involved in the allogeneic rejection reaction. Sponges in general (Simpson, 1984), and *C. diffusa* specifically (Smith and Hildemann, 1986a, b; 1988), have fewer than a dozen cell types. The suggestion that gray cells, which are present in most sponge species (Boury-Esnault, 1977), may serve an immune function (Humphreys, 1994) provides insight into the nature of this cell type, whose function has otherwise been unknown (Simpson, 1984). Identifying a specific cell type found in many species of sponges as a possible immunocyte will provide focus for cellular and molecular studies of immune function in sponges.

The following aspects of our results will be discussed more fully: (1) the basis for the more rapid, 48-hour reactions of the tissue fragments on insect pins, and of allogeneic mixed cells in aggregates, relative to the one-week reactions of intact tissues; (2) the nature of the reactions that inhibit cell aggregation of the allogeneic cell mixtures; and (3) The processes occurring in newly formed, individual-specific aggregates that shorten the time to cytotoxic reactions from 36 hours of allogeneic contact to 8 hours and allow the rapid accumulation of gray cells at the allogeneic boundary.

48-hour reactions

When intact fingers of *C. diffusa* are apposed, cytotoxic reactions are not detectable until about one week later (Hildemann *et al.*, 1980, and our own experiments). In contrast, the cytotoxic reactions in the insect pin assay or within aggregates of dissociated cells begin in less than 48 hours. We propose that wounding or dissociation of the tissue quickly releases rate-limiting steps to the initiation of the sponge immune response, a process that requires several days when activated by allogeneic contact signals alone. The biological purpose for rate-limiting steps may be to delay as much as possible the nonspecific cell killing, which destroy the cells of the reacting animal as well as those of the foreign organism (Bigger *et al.*, 1981). Thus, the sponge seems to have mechanisms that respond slowly against foreign contacts when they minimally impinge on the sponge tissue, but can react vigorously to foreign contacts when tissue damage is involved. The more rapid second-set reactions that have been described in *C. diffusa* (Hildemann *et al.*, 1980) may be related to this acceleration. These authors noted that for about three weeks after undergoing a rejection reaction,

a finger of *C. diffusa* will react more rapidly to a second allogeneic contact.

Inhibition of aggregation

The inhibition of aggregation, which is manifested in an allogeneic mixture of dissociated cells within an hour of first contact, appears to be a specific response to non-self recognition upon allogeneic contact. We believe that aggregation slows because cell movement is suppressed, possibly by a chemokine released as part of the signaling of non-self recognition. In the experimental setting, a reduction in cell motility would slow the formation of larger cell clumps, because cell movement is required to stabilize nascent attachments between two cell clumps when they happen to make contact in the shaking suspensions. Instead of cells moving so that the initial adhesion spreads and becomes more stable, the two clumps adhere only at the point of initial contact and are ultimately sheared apart again by the motion of the liquid. Indeed, if shaking is stopped the allogeneic aggregates will adhere but do not tend to form a single sphere, a process requiring cell movement (Steinberg, 1963). Likewise the failure of two aggregates in allogeneic contact to round up into one spherical aggregate, even though they remain adherent, confirms that cell movement has been suppressed (Steinberg, 1963).

Studies with an individual specific monoclonal antibody (Smith and Hildemann, 1986b) show that the cells of two parabiosed fingers of *C. diffusa* do not mix during the several days they are in contact before the cytotoxic reaction begins. Because sponge cells usually migrate constantly, and visual observation of the adhesion between the cells of the surfaces of two parabiosed sponge fingers establishes that they are in intimate contact, the failure of the cells of the two contacting sponges to mix would be surprising if cell movement were active. *In vivo* the suppression of cell movement upon contact with non-self tissue may functionally reduce the potential for parasitic or pathogenic cells to enter the sponge tissue during the early phases of allogeneic contact before the cytotoxic rejection reactions begin.

8-hour reactions

The ability of 48-hour aggregates to carry out the cell killing reaction within 8 hours of allogeneic contact indicates that tissue and cells are able, under properly stimulated conditions, to progress from the primary allogeneic recognition event to the cytotoxic effector activity in only 8 hours. We have also suggested that suppression of aggregation in allogeneic mixtures of cells reflects a signal from primary allogeneic recognition and occurs within one hour of allogeneic cell contact. This signal, which suppresses cell movement, is evident in one hour even in cells that are freshly isolated from intact tissue

and that require 36 hours to react with cytotoxicity. If these suggestions are valid, the slowness of the cells to carry out allogeneic recognition *per se* is not the limiting factor in the slower reactions. Rather, the delay seems related to the ability of the immune process to proceed to the cytotoxic effector function in response to allogeneic recognition signals. The striking observation that the gray cells in 48-hour aggregates are rapidly mobilized suggests that the responsiveness of gray cells may be one of the accelerated parameters. The acceleration in the sponges of alloreactivity upon wounding or disruption of the tissue may be conceptually related to the idea in the vertebrate immune system that there are a variety of "danger" signals that make the animal more likely to react to a given antigen as foreign (Matzinger, 1994). In *C. diffusa*, these "danger" signals produced from tissue wounding accelerate cytotoxic reactivity from a time course of 170 hours to one of 8 hours.

We suggest above that the inhibition of aggregation may be a response of cells to the release of a chemokine at the boundary of allogeneic contact. We have demonstrated that gray cells are induced to move to the zone of allogeneic contact, a response analogous to those guided by chemokines generated during the reactions of cells in the vertebrate immune system. In vertebrates, there are many chemokines, each with a variety of functions. Thus, from this model one may suppose that one, or more, chemokines may well act to inhibit general cell movement, preventing the ingress of foreign cells, and to attract gray cells to move through these inhibited cells to the boundary of allogeneic contact.

An overview of the immune reaction of C. diffusa

Superficial contact of *C. diffusa* with non-self tissue or cells is quickly recognized and leads to the production of a signal that suppresses the movement of self cells in the zone of contact. This suppression may reduce the entry of foreign cells into the sponge tissue. If the contact persists, or if contact involves significant tissue damage, an acceleration of the immune process occurs, and gray cells become more responsive to the signal to move to the boundary of allogeneic contact. As the gray cells accumulate at the zone of allogeneic contact, cell killing effector mechanisms are initiated, and all cells in the zone of contact, both foreign and self, are killed. This later response, although harmful to the sponge, presumably eliminates any organism that is attacking or trying to gain entry into the sponge tissue. The state of accelerated reactivity of the sponge immune system may last about three weeks.

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Primordial Germ Cells of *Synaptula hydriformis* (Holothuroidea; Echinodermata) Are Epithelial Flagellated-Collar Cells: Their Apical-Basal Polarity Becomes Primary Egg Polarity

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Abstract. The primordial germ cells (PGCs) of a recently metamorphosed juvenile *Synaptula hydriformis* occur with somatic cells in the germinal epithelium of the gonad. As part of the epithelium, PGCs rest on a basal lamina, extend apically towards a lumen, are joined to other cells of the epithelium *via* apicolateral junctions, and express apical-basal polarity. Each PGC has an apical flagellum that is surrounded by a collar of microvilli. The apicolateral junctions of PGCs consist of apical adhering and subapical septate junctions. Hemidesmosomes attach the PGCs to the basal lamina. Although the somatic cells form an incomplete layer over the PGCs, both the PGCs and somatic cells remain exposed to the apical lumen and retain contact with the basal lamina. The peritoneum is the outermost layer of the gonad and faces the perivisceral coelom. The epithelial-cell characteristics expressed by cells of the peritoneum are identical to those of the germinal epithelium. PGCs of *S. hydriformis* are epithelial flagellated-collar cells and express the apical-basal polarity that is typical of epithelial cells. The apical-basal polarity of the oocyte, animal-vegetal axis of full-grown eggs, and anterior-posterior axis of larvae and adults are all in correspondence. Thus the polarity of the germinal epithelium may determine the primary body axis of the next generation.

Introduction

Most eggs have an inherent polarity, expressed as the animal-vegetal axis, that is visible at least by the eccentric nucleus and site of production of polar bodies (Wilson,

1896). Although the relationship of primary egg polarity to embryonic polarity has been scrutinized in a number of species, and the genetic control and expression of both egg and embryo polarity are currently under investigation in a few species, a general model of the origin of egg polarity has not been forthcoming. Thus, although E.L. Mark suggested in 1881 that egg polarity was determined by “the topographical relation of the egg (when still in an indifferent state) to the remaining cells of the maternal tissue from which it is differentiated” (Mark, 1881, p. 515), his hypothesis has never been explicitly tested.

Current research on egg polarity centers primarily on the asymmetric distribution within the egg of maternally derived determinants (see Davidson, 1986, for general information; Gard, 1995, for *Xenopus*; González-Reyes *et al.*, 1995, and Curtis *et al.*, 1995, for *Drosophila*). The initial, spatial relationship of the oocyte to maternal tissues, a possible cause of these asymmetries, has been examined in relatively few species. In *Drosophila melanogaster*, it has been shown that both larval axes (anterior-posterior and dorsal-ventral) are determined by interactions between the oocyte and cells of the maternal egg chamber, resulting in the localization of specific, maternally derived mRNAs (González-Reyes and St. Johnston, 1994; González-Reyes *et al.*, 1995; Curtis *et al.*, 1995). In *Xenopus laevis*, the animal-vegetal axis of the oocyte and egg is determined during oogenesis, and the anterior-posterior embryonic axis “can be roughly superimposed on [it]” (Gard, 1995). Animal-vegetal polarity is hypothesized to be inherent in the oocyte, rather than the result of its spatial relationship to follicular tissue (reviewed in Gerhart *et al.*, 1983; Gard, 1995), but the establishment of dorsality is related to the point of

sperm entry and rotation of egg contents (Gerhart *et al.*, 1989). Echinoderm eggs also become polarized along the animal-vegetal axis during oogenesis (Boveri, 1901a, b; Jenkinson, 1911; Hörstadius, 1973; Maruyama *et al.*, 1985), and the relationship of the oocyte to maternal tissue is thought to establish animal-vegetal egg polarity (Schroeder, 1980a, b, 1985, 1986; Smiley, 1988).

One obstacle to understanding the relationship between the oocyte and maternal tissues has been the difficulty in identifying a marker of polarity that is present in the oocyte before it is released from the tissue of the ovary (Mark, 1881). "What is needed is to trace the polarity back to its earliest manifestation and to discover some ultrastructural [or other marker]" (Schroeder, 1980a) that is visible while the oocyte-somatic tissue relationship can be documented.

Holothuroid oocytes are visibly polarized cells. The animal pole is identified by a structure termed the apical protuberance (first used by Smiley, 1988) and an apically displaced nucleus. The protuberance is an area of yolk-deficient ooplasm that protrudes from the surface of the oocyte, and is visible using both light microscopy and electron microscopy (Smiley and Cloney, 1985; Smiley, 1988; Frick *et al.*, 1996). Polar bodies are produced at the apical protuberance (Maruyama, 1980; Smiley and Cloney, 1985). Because the protuberance develops while the holothuroid oocyte is still associated with other cells and tissues of the ovary, yet remains visible in the spawned, mature egg as either the protuberance itself (Smiley and Cloney, 1985) or as a defect in the jelly layer (Frick, unpub. data), the polarity of the spawned egg can be related to that of the oocyte within the ovary.

Using the apical protuberance and apically displaced nucleus as markers, we showed that the animal-vegetal polarity of the full-grown oocyte of *Synaptula hydriformis* corresponds with the apical-basal polarity of the parental germinal epithelium (Frick *et al.*, 1996). It has also been reported that the polarity of *Parastichopus californicus* oocytes is identical to epithelial polarization (Smiley, 1988). By tracing morphogenesis from oogonia to full-grown oocytes in *S. hydriformis*, we determined that the polarity of the egg, at all stages of morphogenesis, parallels the polarity of the germinal epithelium. Because gonial cells and previtellogenic oocytes also expressed structures typical of epithelial cells, such as an apical rudimentary flagellum, we hypothesized that the germ cells might be derived from epithelial cells (Frick *et al.*, 1996).

Epithelial cells, in general, rest on a basal lamina and form junctions (hemidesmosomes) with it; they extend apically towards a lumen or space; they form specialized, apicolateral, junctional complexes (among invertebrates, usually a zonula adherens and a septate junction) with neighboring cells; and they express apical-basal po-

larity, as indicated by, for example, an apical flagellum (Welsch and Storch, 1976; Hay, 1990; Ruppert and Barnes, 1994).

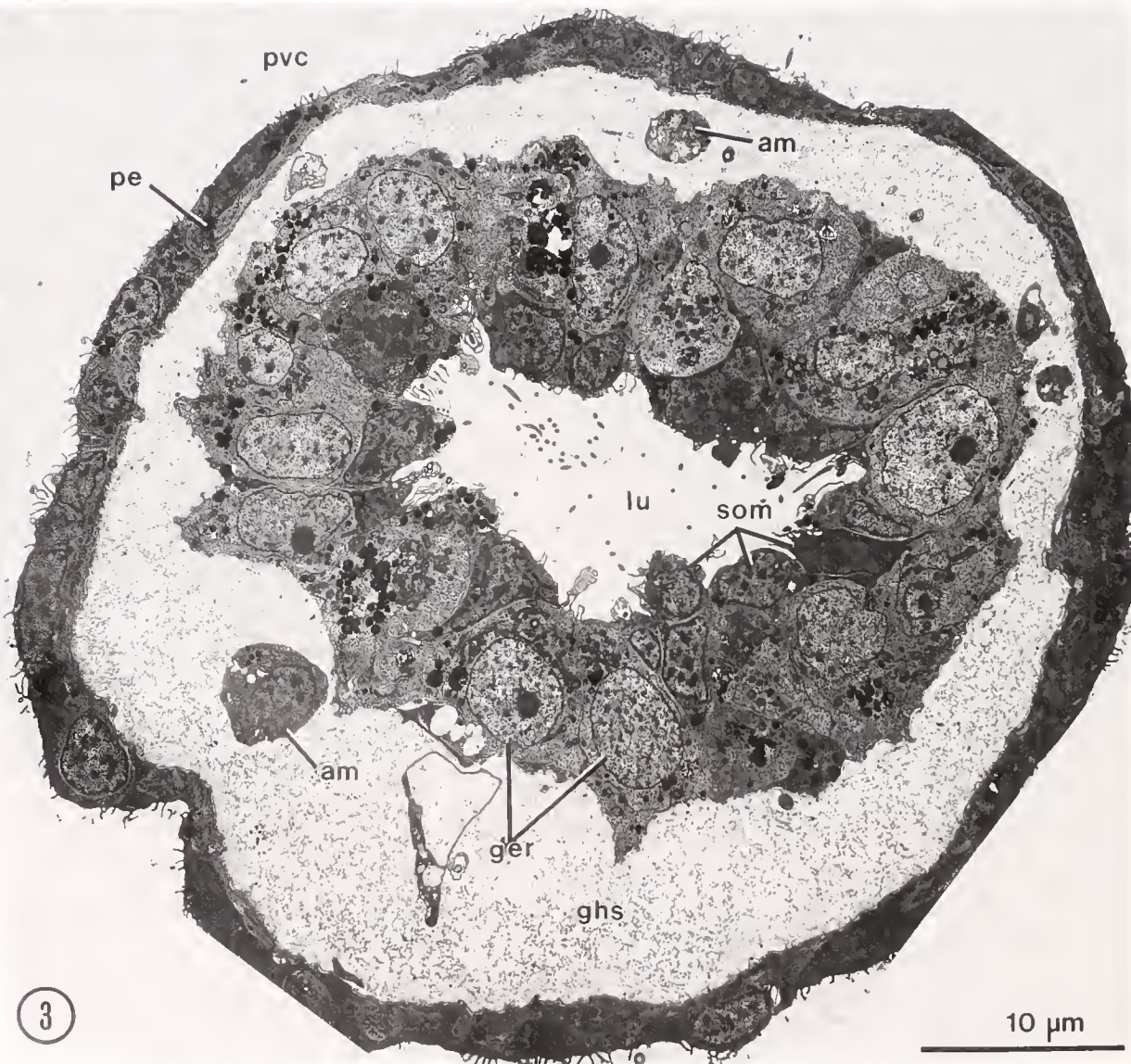
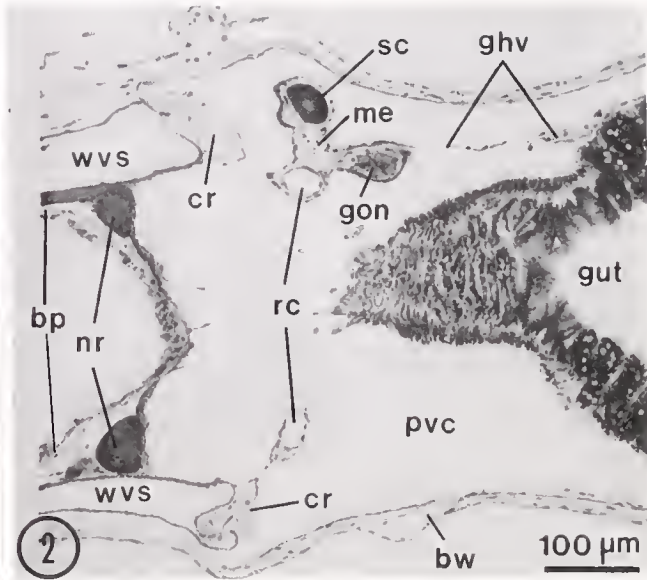
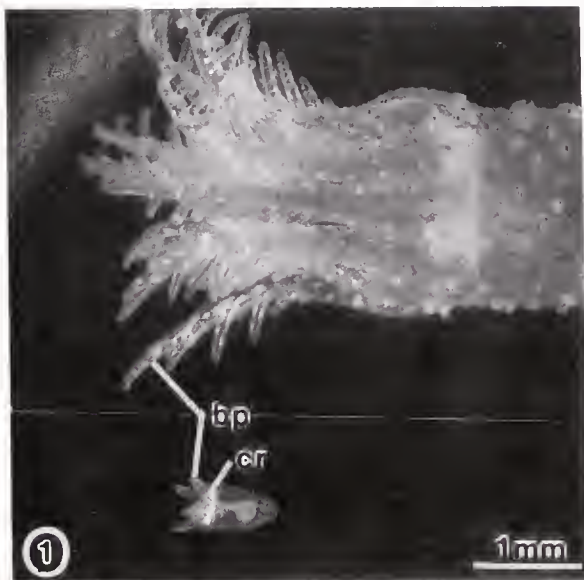
Primordial germ cells (PGCs) are defined by Wilson (1896) as the first germ cells that are "clearly distinguishable from the somatic cells." PGCs are sexually indifferent and capable of migration; gonial cells are sexually determined and nonmigratory (Wilson, 1896; Wourms, 1987). Both PGCs and gonial cells divide by mitosis (Wourms, 1987). According to these definitions, PGCs and gonial cells are differentiated on the basis of genetics and behavior, not structure. In the absence of any structural criteria with which to distinguish between PGCs and gonial cells, we consider the germ cells of juvenile *Synaptula hydriformis* to be PGCs, rather than oogonia, because the juvenile is recently metamorphosed and the gonad is rudimentary. In the adult gonad (Frick *et al.*, 1996), we considered the population of morphologically similar cells that contained dividing cells to be oogonia and spermatogonia.

Nuage, or germ plasm, is considered to be a marker of germ cells (Eddy, 1975). Nuage is known to occur in echinoderm germ cells (Eddy, 1975); in the holothuroid *Parastichopus californicus*, nuage does not appear in the PGCs until 6 months after metamorphosis (Smiley, 1988), but in the echinoid *Lytechinus pictus*, nuage appears in the PGCs of 3-week postmetamorphic urchins (Houk and Hinegardner, 1980). In *Synaptula hydriformis*, nuage has been described from previtellogenic oocytes (Frick *et al.*, 1996). It appears in the perinuclear region of the cytoplasm as one or more clumps of amorphous, electron-dense, non-membrane-bound material that is frequently associated with mitochondria (Frick *et al.*, 1996).

We have undertaken this study of the ovotestis of a recently metamorphosed juvenile of *Synaptula hydriformis* to determine whether PGCs are epithelial cells and if their polarity corresponds to that of the germinal epithelium. If so, we predict that PGCs (in this species destined to become *both* spermatogonia and oogonia) should express the characteristics of typical invertebrate epithelial cells, as noted above. A demonstration that PGCs are epithelial and polarized would imply that both sperms and eggs are, at least primitively, intrinsically polarized cells. In the case of eggs, this polarity would manifest itself as animal-vegetal, primary egg polarity.

Materials and Methods

Adult specimens of *Synaptula hydriformis* were collected in Lake Surprise, Key Largo, Florida, and transported to the Smithsonian Marine Station in Fort Pierce, Florida (described in Frick *et al.*, 1996). Brooded juveniles were removed from the adult perivisceral coe-



lom, relaxed in isosmotic MgCl_2 mixed equally with seawater, and fixed. Primary fixation was at room temperature in isosmotic 2.5% glutaraldehyde in Millonig's 0.2 M phosphate buffer for 1.5 h; secondary fixation was in isosmotic 1% osmium tetroxide in Millonig's 0.1 M phosphate buffer for 1 h. Whole juveniles were dehydrated in a graded series of ethanols and embedded in epoxy resin. Before sectioning, the body wall, which contained calcareous ossicles, was trimmed from the block. Sections were cut on an MT2B ultramicrotome. Thick sections for light microscopy were stained with 1% methylene blue and 1% azure II and photographed with a Zeiss photomicroscope III. Thin sections for transmission electron microscopy were stained with 6% alcoholic uranyl acetate and Reynold's lead citrate and were photographed with a Zeiss EM9S2. Living adults and juveniles were photographed with a Nikon macro lens.

Results

Brooded, recently metamorphosed juveniles of *Synaptula hydriformis* have five buccal podia, a calcareous ring, and a gut that are visible externally or through the transparent body wall (Fig. 1). In sectioned animals, ampullae of the buccal podia, other parts of the water-vascular system, the circumesophageal nerve ring, the calcareous ring, and the gut are visible (Fig. 2). The gonad is present in juveniles and is lodged in the dorsal mesentery, as are the stone canal and ring canal of the water-vascular system (Fig. 2). A hemal sinus surrounds the gonad and is joined to the hemal system of the gut by a hemal vessel that passes through the mesentery (Fig. 2).

The juvenile gonad consists of three layers (Fig. 3). An outer peritoneum faces the perivisceral coelom, an inner germinal epithelium surrounds the gonadal lumen, and a connective-tissue layer is situated between the two epithelia.

The connective-tissue layer and the genital-hemal sinus occupy the same tissue compartment, and structural connective tissue grades into hemal fluid. Immediately beneath the peritoneal basal lamina, a few collagen fibers are present, but slightly deeper into the connective tissue,

collagen is replaced by the abundant proteins of the hemal fluid (Fig. 3). Cells within the genital-hemal sinus are amebocytes (Fig. 3), as described from the adult gonad (Frick *et al.*, 1996).

The simple germinal epithelium is composed of germinal and somatic cells. The germinal epithelium of adult *Synaptula hydriformis* produces both sperm and eggs (see Frick *et al.*, 1996); thus the PGCs of the juvenile gonad differentiate into oogonia and spermatogonia. The germ cells are more or less cuboidal in shape and make the epithelium a thick, 10–15- μm layer (Fig. 3). The germinal and somatic cells are visibly distinct from one another (Fig. 4).

PGCs have a large, 7- μm circular nucleus with chromatin distributed in sparse clumps and a prominent nucleolus (Figs. 3–8). Their diffuse but uniform cytoplasm stains more lightly than that of the somatic cells (Figs. 3–8). PGCs contain nuage (Figs. 7, 8). Mitochondrial profiles are common throughout the cytoplasm (Figs. 3–8). Golgi bodies are primarily apical in position (Figs. 5–7). Dark, lipoidal inclusions, which are not membrane-bound, are found in both PGCs and somatic cells (Figs. 3–10).

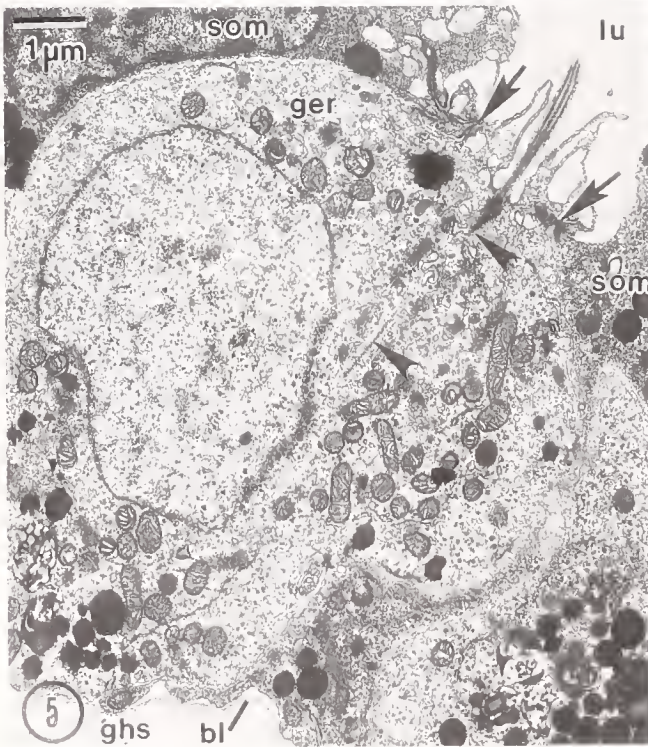
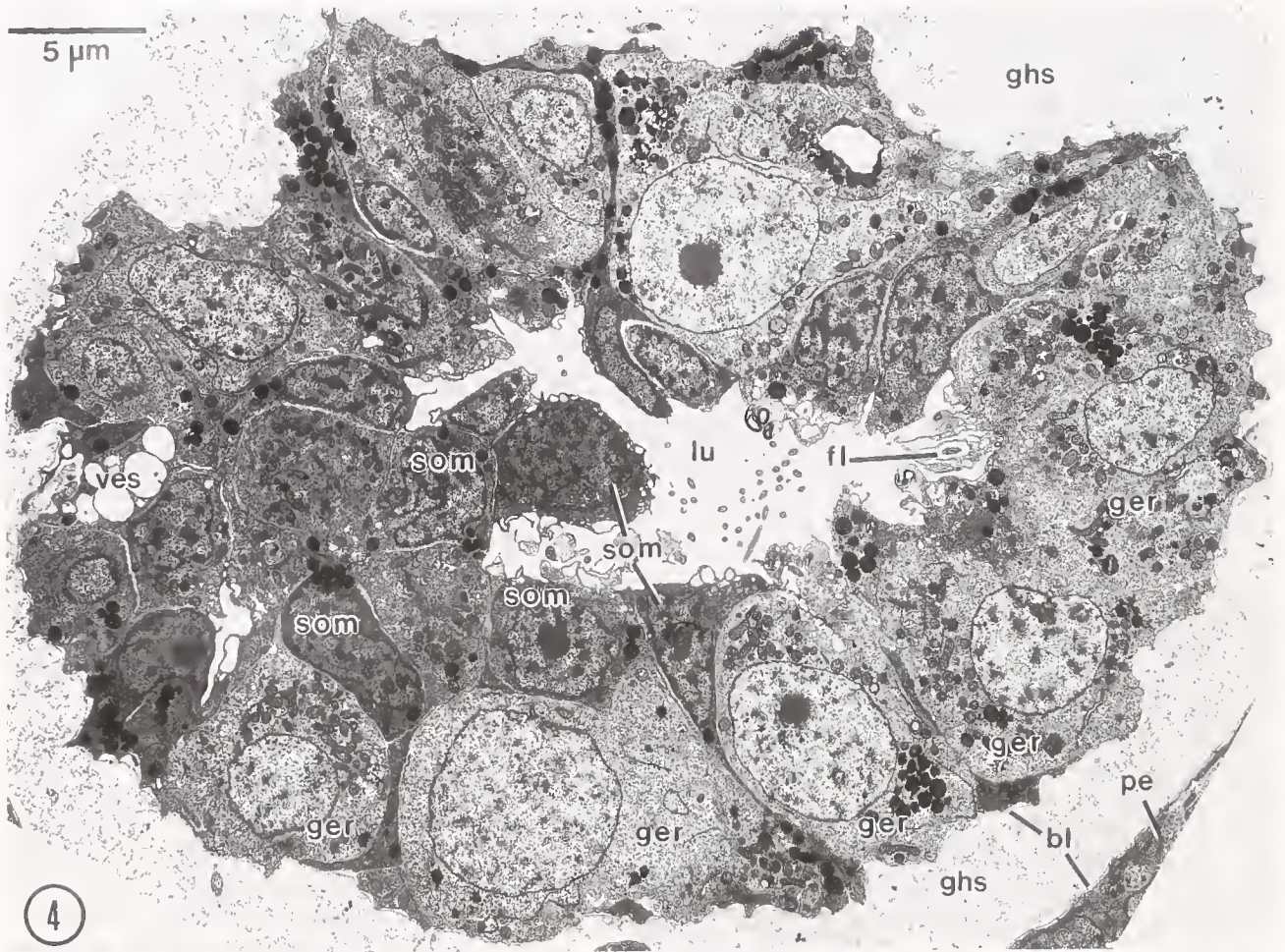
PGCs display characteristics of epithelial cells. They are part of a sheet of cells that rests on a common basal lamina (Figs. 3, 4), they are joined by intercellular junctions to other cells (Figs. 5, 6), and the cells express an apical-basal polarity (Figs. 5, 6). Each PGC has an apical flagellum that extends into the lumen of the tubule (Figs. 5, 6). The flagellum is surrounded by a collar of microvilli (Figs. 5, 6), and a striated rootlet fiber anchors it to the nuclear membrane (Fig. 5). The Golgi bodies noted above are frequently associated with the flagellar base (Figs. 5–7). The apicolateral junctions of PGCs are complex and consist of an apical adhering junction (probably a zonula adherens) and a subapical septate junction (Fig. 6 inset). Hemidesmosomes join the PGCs to the basal lamina on which they rest (Fig. 7 inset).

The perikarya of the somatic cells of the germinal epithelium form an incomplete layer over the PGCs, partially separating them from the apical lumen and the basal lamina (Figs. 3–4, 8). Part of the apical surface of

Figure 1. Adult and juvenile *Synaptula hydriformis* (photomacrograph). The juvenile was removed from the perivisceral coelom of the adult. Buccal podia (bp), calcareous ring (cr).

Figure 2. Longitudinal section of juvenile (light micrograph). The nerve ring (nr), calcareous ring (cr), and ring canal (rc) encircle the esophagus (not in plane of section). The gonad (gon) is embedded in a mesentery (me) that extends from the ring canal (rc) to the gut (gut). Within this same mesentery, a hemal vessel (ghv) and the stone canal (sc) also occur. Buccal podia (bp), body wall (bw), perivisceral coelom (pvc), water vascular system (wvs).

Figure 3. Transverse section of juvenile gonad (transmission electron micrograph). The peritoneum (pe) faces the perivisceral coelom (pvc). The genital-hemal sinus (ghs) contains amebocytes (am) and proteins. The germinal epithelium is composed of both germinal (ger) and somatic (som) cells and faces the gonadal lumen (lu).



each PGC, however, is exposed to the lumen, and part of the basal surface rests on the basal lamina (Figs. 4–6). The perikaryon of the somatic cell is displaced apically, but the cell remains in contact with the basal lamina *via* slender cellular processes that pass between the germ cells (Figs. 4, 7, 8). Thus, the germinal epithelium is a simple, but pseudostratified, epithelium composed of germ and somatic cells. The nuclei of somatic cells are smaller than those of germinal cells (Fig. 4). They are irregularly shaped and contain discrete patches of heterochromatin, much of which is peripherally located (Figs. 3–8). They may also possess a nucleolus (Figs. 3, 4). The dense cytoplasm of somatic cells stains more darkly than that of germ cells (Fig. 3–8). Although some mitochondria are present (Figs. 4, 6), few other structures except lipoidal granules are apparent (Figs. 4–8).

The peritoneum of the gonadal tubule is a flattened epithelium, *ca.* 2- μ m thick (Figs. 3, 9–11), that rests on a basal lamina and faces the perivisceral coelom. Nuclei of these somatic epithelial cells are flattened in the plane of the epithelium and contain patches of peripheral heterochromatin (Figs. 3, 9). The cytoplasm, which is moderately dense, contains numerous mitochondria, cisternae of rough endoplasmic reticulum, and lipoidal granules (Figs. 9–11). An apical flagellum, originating from a basal body anchored with a rootlet fiber, extends into the perivisceral coelom and is surrounded by a collar of microvilli (Fig. 9). Microvilli also occur elsewhere on the apical surface of the epithelial cells (Figs. 9, 10). Hemidesmosomes attach the epithelial cells to the basal lamina (Fig. 10). Both longitudinal (Fig. 10) and circular (Fig. 11) muscle fibers, as well as nerves (Figs. 9, 11), occur in the peritoneum. Cells of the peritoneum are joined in a junctional complex identical to that of the germinal epithelial cells. An apicolateral adhering junction underlain by a septate junction joins adjacent cells (Fig. 12).

Discussion

PGCs as epithelial cells

As shown in this study, the PGCs of *Synaptula hydriformis* express all the characteristics of typical invertebrate epithelial cells. These characters are identical to those expressed by epithelial cells of the perivisceral peri-

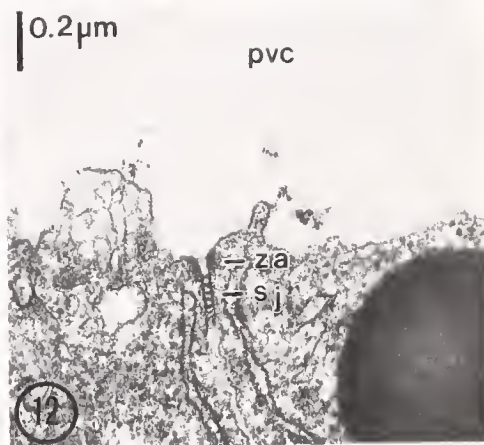
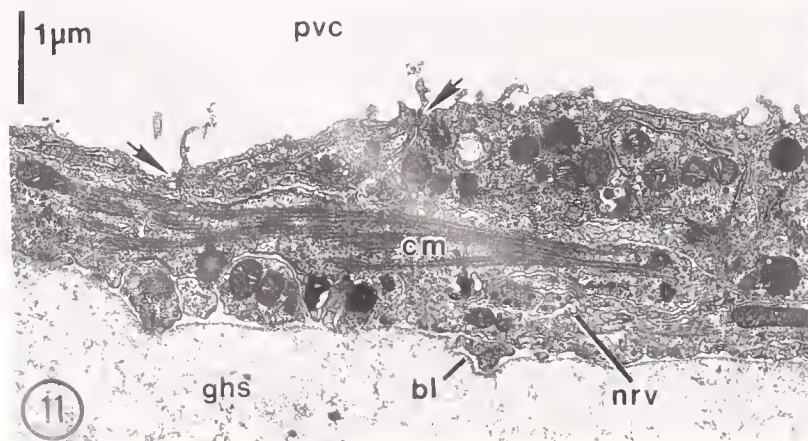
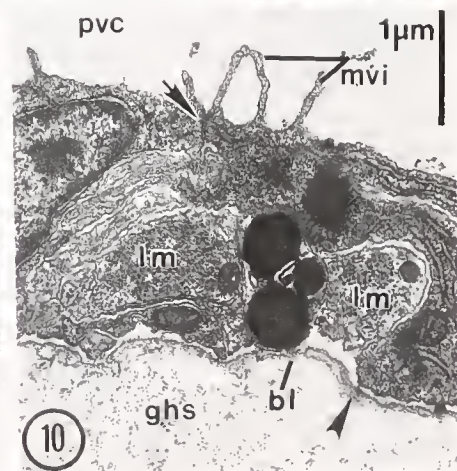
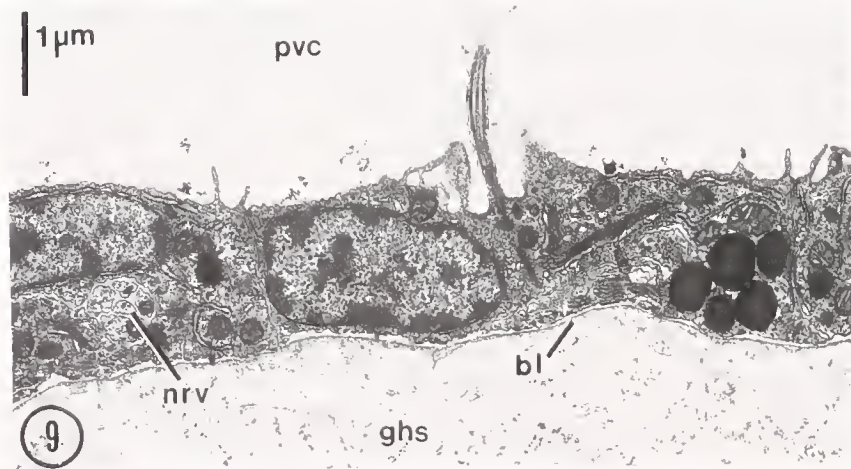
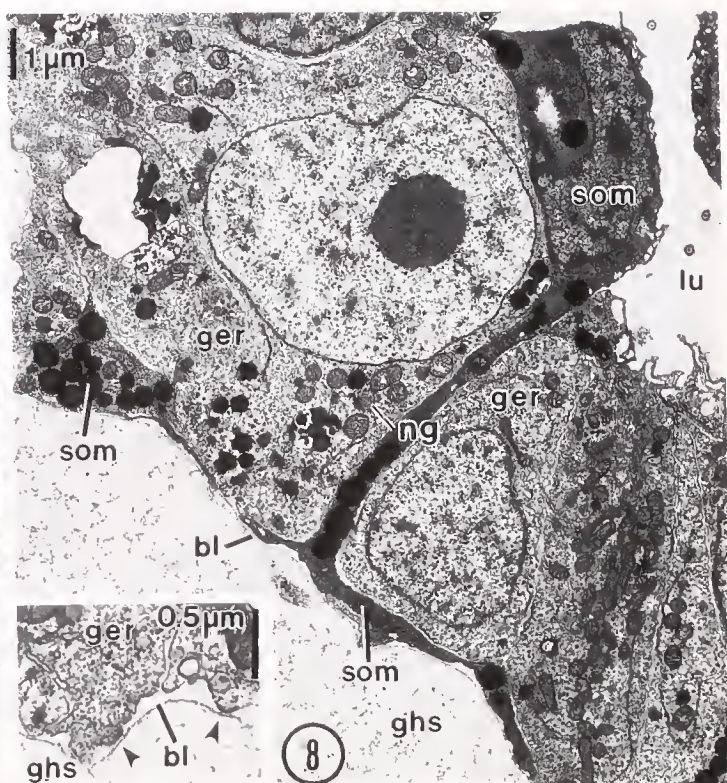
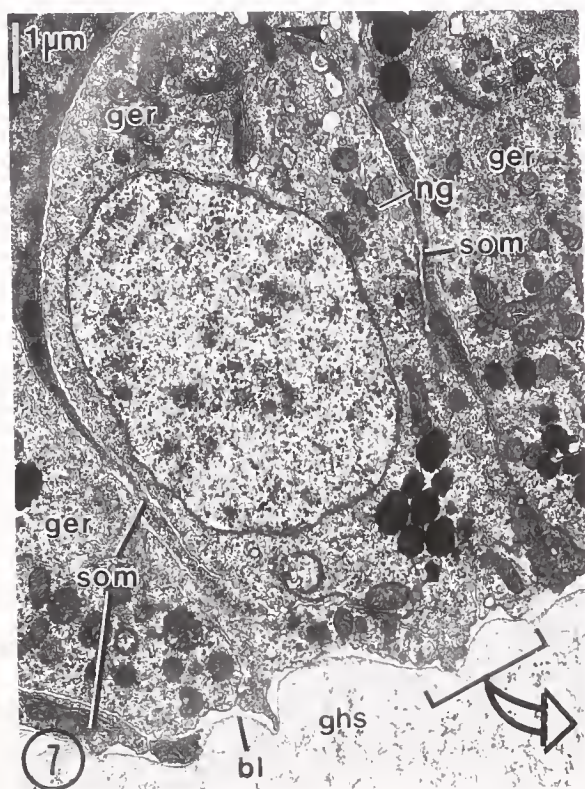
toneum, leaving little doubt that the PGCs are epithelial cells. This is the first complete demonstration of the epithelial nature of echinoderm PGCs.

Two other electron-microscopic descriptions of echinoderm PGCs identify some, but not all, epithelial characteristics. In the holothuroid *Parastichopus californicus* (Smiley, 1988), PGCs, oogonia, and previtellogenic oocytes are “attached to the . . . basal lamina,” although no hemidesmosomes are reported, and they extend towards the lumen of the tubule. The cells are bound to other cells with intercellular junctions, described as zonulae adhaerentes. PGCs have apical centrioles and “appear to bear a cilium”; a single previtellogenic oocyte was shown to have an apical centriole (Smiley, 1988). Smiley (1988) recognized that these oocyte characteristics were specific to epithelial cells. Our report confirms and expands on his observations. Junctions with neighboring cells, association with basal lamina, and an apical flagellum are variously noted in the previtellogenic or early vitellogenic oocytes of several other species of holothuroids (Eckelbarger and Young, 1992; Tyler *et al.*, 1994; Frick *et al.*, 1996).

The PGCs of echinoids also express some characteristics of epithelial cells. Houk and Hinegardner (1980) reported intercellular “tight junctions” between somatic cells and PGCs and “junctional complexes” between PGCs. They also noted “striated rootlets and flagellar bases” that, though regionally unspecified, are presumably apical in position. The PGCs and oogonia rest on a basal lamina along with other cells of the epithelium (Houk and Hinegardner, 1980; Frick and Ruppert, unpub. data). Furthermore, on the basis of their similar morphology, the PGCs and somatic accessory cells are thought to be derived from a common embryological origin. Both PGCs and somatic cells contain “membrane-bound vesicles filled with an electron-dense material which resembles yolk” (Houk and Hinegardner, 1980), perhaps similar to the lipoidal inclusions seen in *Synaptula hydriformis* PGCs and somatic cells.

The expression of a collared flagellum in the PGCs of *Synaptula hydriformis* (Figs. 5, 6) is significant for two reasons. First, it indicates that the PGC, perhaps after a migratory period, differentiates initially into a specialized cell, *viz.*, an epithelial flagellated-collar cell. Second,

Figures 4–6. Transverse sections of the germinal epithelium (transmission electron micrograph). Fig. 4: Germinal (ger) and somatic (som) cells of the germinal epithelium rest on the basal lamina (bl) and extend into the gonadal lumen (lu). Flagellum (fl), genital-hemal sinus (ghs), peritoneum (pe), vesiculated somatic cell (ves). Figs. 5–6: The base of germinal cells (ger) rests on a basal lamina (bl) and the apex, with a flagellum surrounded by microvilli, extends to the lumen (lu). The cells have apicolateral junctions (arrows) with other cells of the epithelium. The rootlet of the flagellum (arrowheads) is anchored to the nuclear membrane. Inset: The junctional complex between a germinal and somatic cell consists of an apicolateral zonula adherens (za) and a septate junction (sj) just basal to it.



because the full-grown eggs lack a flagellum, oogenesis is a partial departure, or dedifferentiation, from a differentiated state. The departure is only partial, however, because the egg retains its epithelial polarity as its animal-vegetal axis despite the loss of flagellum, junctions, and shape of a typical epithelial cell (Frick *et al.*, 1996). Thus, the PGCs of *S. hydriformis* are not a reserve of undifferentiated cells but somatically differentiated cells with the capacity to become germ cells.

On the basis of their position within the dorsal mesentery and their morphology, echinoderm PGCs are claimed by some to be mesenchymal (Hamann, 1888; Delavault, 1966; Houk and Hinegardner, 1980; Smiley, 1988), while others classify them as epithelial (Russo, 1902; MacBride, 1936; Nieuwkoop and Sutasurya, 1981; Holland, 1991; Hendler, 1991). This report does not identify the origin of presumptive PGCs in the development of *S. hydriformis*, but does indicate that at the time the PGCs are lodged in the gonad they are epithelial cells; this observation is substantiated by evidence from other holothuroids (Smiley, 1988) and echinoids (Houk and Hinegardner, 1980). Although Houk and Hinegardner (1980) classify echinoid PGCs as mesenchymal, their report shows that the PGCs rest on a basal lamina, form junctions with other cells, and possess components of a flagellum. These are all characteristics of epithelial, not mesenchymal, cells. Similarly, Smiley (1988) describes epithelial characteristics for holothuroid PGCs as discussed earlier, but identifies presumptive PGCs as mesenchymal cells because they are lodged initially in connective tissue. Because epithelial-mesenchymal transitions occur among somatic cells (Welsch and Storch, 1976; Hay, 1990), the possibility remains that the germ cells segregate initially as mesenchymal cells but transform into epithelial cells after incorporation into the gonad.

Origin of egg polarity

We have demonstrated that egg polarity in *Synaptula hydriformis* parallels the polarity of the maternal germi-

nal epithelium (as has Smiley, 1988, for *Parastichopus californicus*). The cells of the epithelium, whether destined to form germ cells or somatic cells, are epithelial cells with an apical-basal polarity. We propose that egg animal-vegetal polarity, therefore, is ultimately derived from the apical-basal polarity of the maternal epithelium. We have not experimentally tested this hypothesis by manipulating developing PGCs or oocytes, but we have demonstrated that animal-vegetal egg polarity in *S. hydriformis* can be traced from the PGC (data herein) to the mature oocyte (Frick *et al.*, 1996) and that it corresponds to apical-basal epithelial polarity at all stages.

Echinoderm eggs are polarized cells, and this primary polarity is established during oogenesis. In several species of echinoids, Schroeder used the jelly canal, polar-body extrusion, and pigment patterns of the egg to identify the primary egg axis (Schroeder, 1980a, b). He hypothesized that the relationship of the egg to maternal tissues caused polarity (Schroeder, 1980a). The apical jelly canal, a channel through the jelly coat (Schroeder, 1980b), is the earliest marker of polarity to appear, and it may be derived from an association with maternal tissue (Schroeder, 1980a, b). Similarly, asteroid oocytes also express an animal-vegetal polarity that can be identified by apical centrioles (Schroeder, 1985; Kato *et al.*, 1990; Schroeder and Otto, 1991), an apically displaced nucleus, an absence of large vacuoles and actin-filled spikes at the animal pole, and a local mechanical weakness at the animal pole (Schroeder, 1985). Holothuroid eggs, as already discussed, express animal-vegetal polarity based on the apical protuberance, apically displaced nucleus (Smiley and Cloney, 1985; Smiley, 1988; Frick *et al.*, 1996), and apical centriole (Smiley, 1988) or flagellum (Frick *et al.*, 1996).

The animal-vegetal polarity of echinoderm eggs and embryos corresponds generally to larval anterior-posterior polarity. This correspondence has been best documented in echinoids by using techniques that include morphological observation, experimental manipulation, and molecular genetic analysis. Boveri (1901b) described

Figures 7–8. Germinal (ger) and somatic (som) cells of the germinal epithelium (transmission electron micrograph). Genital-hemal sinus (ghs), nuage (ng). Fig. 7: Both germinal and somatic cells make contact with the basal lamina (bl). Flagellum (arrowhead). Inset: Germinal cells form hemidesmosomes (small arrowheads) where they contact basal lamina (bl). Fig. 8: Somatic-cell nuclei may partially separate germinal cells from the gonadal lumen (lu), and extensions of the somatic cells may extend to the basal lamina (bl) and partially undercut germinal cells.

Figures 9–12. Cells of the peritoneum (transmission electron micrograph). Fig. 9: Flagellated epithelial cells and nerves (nrv) occur in the peritoneum. The cells rest on a basal lamina (bl) that separates them basally from the genital-hemal sinus (ghs). Apically, they extend into the perivisceral coelom (pvc). Fig. 10: Longitudinal muscle fibers (lm) are present in the epithelial cells and microvilli (mvi) occur apically. Hemidesmosomes (arrowhead) connect cells to the basal lamina and apicolateral junctional complexes (arrow) connect cells to one another. Fig. 11: Circular muscle fibers (cm) are present in epithelial cells. Fig. 12: The apicolateral junctional complexes are composed of a zonula adherens (za) and septate junction (sj).

the position of a circumferential pigment band present in the oocytes, eggs, embryos, and larvae of *Paracentrotus lividus*. Relative to the apical jelly canal (micropyle), the pigment band is subequatorial, in the vegetal half of the egg. The pigment band remains vegetally located throughout embryonic development and eventually moves in through the blastopore, at the posterior end of the larva (Boveri, 1901b). The vegetal pole of the egg thus becomes the posterior end of the larva. Boveri's results (1901a, b) are supported by the experimental manipulations of Hörstadius (reviewed in 1973) and confirmed by Schroeder's (1980a) observations. A molecular genetic analysis of the establishment of the oral axis in echinoid larvae (Cameron *et al.*, 1989) also confirms that the animal-vegetal axis remains fixed from egg to larva and that the oral axis develops with a specific relationship to the animal-vegetal axis. Thus, the animal-vegetal axis of the echinoderm egg gives rise to the animal-vegetal axis of the embryo, which develops into the anterior-posterior axis of the larva and is itself derived from the apical-basal polarity of the maternal epithelium. The polarity of the maternal epithelium, therefore, corresponds to that of the larva.

A feature of larval polarity unique to holothuroids is that the larval anterior-posterior axis is identical to the adult anterior-posterior axis (Smiley, 1986). In other classes of echinoderms, the adult axis is shifted from that of the larval axis (Smiley, 1986). Thus in holothuroids, which embody plesiomorphic echinoderm developmental characteristics (Smiley, 1986), the adult anterior-posterior polarity is the same as the apical-basal polarity of the germinal epithelium from which the egg arose.

The hypothesis that egg polarity is derived from the polarity of the maternal germinal epithelium, whether a simple epithelium or complex ovary, is neither new nor restricted to echinoderms. It apparently originated with Mark (1881; also Wilson, 1896) and embraced oogenesis in all animals. Mark (1881, p. 515) stated: "If, in cases where the egg is directly developed from epithelial cells," the position of "the germinal vesicle bears a constant relation to the free surface of the epithelium from which the egg takes its origin," then "it would be fair to infer the existence of corresponding, though obscured, relations in those cases where . . . the origin of the ovum is less directly traceable to an epithelial surface."

Although the oocytes of all animals may not derive their animal-vegetal polarity from apical-basal epithelial polarity, the correlation may be widespread and phylogenetically ancient. On the basis of data presented here for echinoderms and elsewhere for cephalochordates and cnidarians (Frick *et al.*, 1996), we advance two hypotheses. The first is that the earliest metazoans were composed of polarized, flagellated cells, some of which differentiated as germ cells and retained their polarity.

The second is that the determinants of primary egg polarity may be related to the factors that establish the polarity of epithelial cells.

Acknowledgments

We thank Dr. Thomas E. Schroeder and Dr. Michael V. Danilchik for stimulating discussions during our stay at the Friday Harbor Laboratories. Two anonymous reviewers provided critical reviews that improved the manuscript. This is contribution 401 of the Smithsonian Marine Station at Link Port. Supported by NSF grant BSR-90-06599 to EER.

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Serotonin Injections Induce Metamorphosis in Larvae of the Gastropod Mollusc *Ilyanassa obsoleta*

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Abstract. Bath-applied serotonin (5-HT) induces competent larvae of the marine snail *Ilyanassa obsoleta* to metamorphose. Previously, the mode of action of 5-HT, whether as an external ligand or as an internal neurotransmitter, was unknown. Larvae were injected with 10^{-4} M 5-HT and other pharmacological agents to provide evidence that serotonergic neurons are necessary for metamorphosis in *Ilyanassa* larvae and that serotonin functions as a neurotransmitter or neuromodulator during this process. About 50% of 5-HT-injected animals metamorphose within 48 hours. Fluoxetine, a 5-HT reuptake inhibitor, and alpha-methyl-5-hydroxytryptamine (α m5HT), a 5-HT agonist, were also effective inducers of metamorphosis. Gramine (3-[dimethylaminomethyl]indole), a 5-HT antagonist, inhibited the inductive activity of 5-HT, while the amino acid gamma-aminobutyric acid (GABA) resulted in rates of morphological restructuring similar to those of controls. Collectively, the results of our experiments support the idea that serotonergic neurons are active during larval metamorphosis of *Ilyanassa* and that 5-HT does not induce metamorphosis by binding to epidermal chemoreceptors.

Introduction

Most marine invertebrates spend the early portion of their lives as planktonic larvae, surviving in an environ-

ment that is drastically different from the benthic one they will inhabit as adults. Larval metamorphosis is often triggered by physical and biological features of the environment. For many animals, especially molluscs, chemical factors are the most important inducers of metamorphosis (reviewed by Crisp, 1974, 1984; Pawlik, 1992a,b). Several molluscs are known to settle and metamorphose in response to specific environmental compounds (Scheltema, 1961; Hadfield, 1977; Morse and Morse, 1984; Hadfield and Scheuer, 1985; Levantine and Bonar, 1986; Zimmer-Faust and Tamburri, 1994). The oyster *Crassostrea virginica*, whose habitat overlaps with that of the mud snail *Ilyanassa obsoleta* (Fox and Ruppert, 1985), appears to settle in response to a conspecifically derived peptide (Zimmer-Faust and Tamburri, 1994). The natural inducer for *Ilyanassa* larvae is unknown, but like the metamorphic inducers for *Crassostrea* and the nudibranch *Phestilla sibogae* (Hadfield and Sheuer, 1985), it is a small, organic, water-borne molecule (Levantine and Bonar, 1986).

Exogenous neurotransmitters can act as external ligands, mimicking the action of natural metamorphic inducers. For example, larvae of the abalone *Haliotis rufescens* are induced to settle and metamorphose by the neurotransmitter GABA and its structural analogues (Morse *et al.*, 1979; Morse and Morse, 1984; Morse *et al.*, 1984). Like the natural inducer, GABA acts at receptors on the larval epithelium (Trapido-Rosenthal and Morse, 1985; 1986a,b; Wodicka and Morse, 1991).

Exogenous 5-HT is a reliable inducer of metamorphosis in *Ilyanassa*, but its mode of action is unclear (Levantine and Bonar, 1986). Levantine and Bonar suggested that 5-HT acts either at a different site or in a manner different from that of the natural inducer. In their experiments, a low level of metamorphosis in response to mud extract (the natural inducer) was reached in

Received 14 July 1995; accepted 28 May 1996.

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Abbreviations. FIO = 0.2- μ m-filtered Instant Ocean; GABA = gamma-aminobutyric acid; 5-HT = 5-hydroxytryptamine (also serotonin); α m5HT = alpha-methyl-5-hydroxytryptamine.

2 hours. The same metamorphic rate was reached 20 hours later for animals exposed to 5-HT. The difference in timing between these two inducers might reflect the time required for uptake and internal release of 5-HT or its conversion to another active molecule within the larval nervous system.

The monoamine serotonin is common in both vertebrate and invertebrate nervous systems (reviewed by Kandel *et al.*, 1991; Frazer and Hensler, 1994). Its role as a neurotransmitter and neuromodulator in a wide range of behaviors in molluscs was extensively reviewed by Walker (1986). As examples, serotonin is active in controlling heartbeat rhythm (Liebeswar *et al.*, 1975) and feeding and biting behaviors (Weiss *et al.*, 1978; Kupfermann and Weiss, 1981; Gelperin, 1981; Ram *et al.*, 1981; 1991; Yeoman *et al.*, 1994), and in modulating swimming motor patterns (Parsons and Pinsker, 1989; McPherson and Blankenship, 1991; Satterlie and Norekian, 1995). It also regulates ciliary beating in larval and adult stages of several molluscan species (Gosselin, 1961; Diefenbach and Goldberg, 1990; Diefenbach *et al.*, 1991; Goldberg *et al.*, 1994). Both freshwater and marine molluscs develop serotonergic neurons as embryos and maintain populations of these neurons throughout their lives (Goldberg and Kater, 1989; Koshtoyants *et al.*, 1961; Diefenbach and Goldberg, 1990; Satterlie and Norekian, 1995), although, as discussed below, populations of serotonergic neurons are not necessarily static (Barlow and Truman, 1992).

Serotonin appears to be an endogenous factor that stimulates ciliary beating in nudibranch veligers (Koshtoyants *et al.*, 1961; Coon *et al.*, 1990; Barlow, 1990). Serotonergic neurons in the CNS innervate the velum I—the swimming and feeding organ—and the foot of the nudibranch *Berghia verrucicornis* (Kempf *et al.*, 1991) and of *Ilyanassa* larvae (S. C. Kempf, Auburn Univ., pers. comm.). This discovery, along with Levantine and Bonar's (1986) result showing that metamorphosis in *Ilyanassa* occurs in response to exogenously applied 5-HT, supports the hypothesis that serotonergic neurons and internally active 5-HT are necessary for larval metamorphosis in this species. The role of 5-HT in settlement and metamorphosis in *Ilyanassa* may or may not be a cilio-excitatory one.

Although the activity of serotonergic neurons may be necessary for the induction of metamorphosis, not all of the serotonergic neurons in the larval brain are retained throughout this process. In *Haliotis*, five pairs of 5-HT-immunoreactive (IR) neurons occur in the cerebral ganglion by four days after fertilization, and one of these pairs innervates the velum (Barlow and Truman, 1992). This particular neural complement remains stable until metamorphosis, when the pair of velar serotonergic neurons is rapidly lost.

As mentioned above, exogenous neurotransmitters,

which are usually small molecules (Erulkar, 1994), may mimic natural compounds by acting at external chemoreceptors (Trapido-Rosenthal and Morse, 1986a,b; Wodicka and Morse, 1991). Larvae can also take up a variety of small molecules from seawater for internal usage (Jaecle and Manahan, 1989). *Haliotis* larvae can transport amino acids from the environment into their bodies, thereby acquiring energy (Manahan, 1983). Transported compounds can affect larval behavior and could even trigger metamorphosis. The delay in response of *Ilyanassa* to 5-HT in comparison to the natural inducer suggests that 5-HT does not act at epithelial chemoreceptors (Levantine and Bonar, 1986). Instead, exogenous 5-HT may be transported across the larval epidermis and be available to mimic the release of endogenous 5-HT and induce metamorphosis. We injected 5-HT and related pharmacological agents into the hemocoel of competent *Ilyanassa* larvae to determine if serotonergic activity in the nervous system promotes metamorphosis.

Materials and Methods

Animal culture

Adult *Ilyanassa obsoleta* (Say, 1922) were kept in the laboratory in well-aerated tanks from which egg capsules were removed twice weekly. Every day capsules were briefly rinsed in 70% ethanol to remove bacteria and were placed in fresh 0.2- μ m-filtered Instant Ocean (FIO). Swimming larvae were removed from the dish and held at 7°C until enough larvae were collected to begin a new culture (Leise, 1996).

Larval *Ilyanassa obsoleta* were cultured in the laboratory from embryos (Collier, 1981) as previously described (Leise, 1996). Briefly, larval cultures were maintained in an airlift system in a 50:50 mixture of 0.2- μ m-filtered natural seawater and FIO with penicillin and streptomycin antibiotics (Miller and Hadfield, 1986). Larvae were fed algae daily: young cultures (less than 3 days old) received 40 ml of *Isochrysis galbana*, *Monochrysis* sp., *Dunaliella tertiolecta*, or *Skeletonema rathbun*; older cultures were also fed *Nannochloropsis* sp. Larval culture medium was changed every 6 days.

General experimental protocol

Experiments in which test solutions were applied to larvae in the bath seawater were conducted in 24-well plastic Falcon tissue culture plates. Ten larvae were added to each well along with 2 ml of the appropriate solution. Each treatment and control group was replicated three times and each experiment was repeated three times, yielding a potential total of 90 individuals. Experimental treatments and control groups for individual experiments were obtained from one culture. Differ-

ent cultures were used for at least two of the repeated experiments to ensure that results were consistent and did not depend upon the culture used. Results from each experiment were pooled, and the combined results were tested for statistical significance, as described below. The graphs presented in this paper were obtained from these pooled results. Mortality was generally near zero in the bath experiments, and around 5% in the injection experiments. The results reported here include only surviving individuals. Usually, less than 400 larvae were available for experimentation in any particular culture. As a result, in many experiments the number of larvae in the control groups was reduced to maintain sufficient numbers of animals in the experimental treatments. This did not impair the statistical significance of the results.

Injection experiments

Animals that attained a shell length of about 630 μm or longer were described as competent by Scheltema (1962). In our cultures, 12–40-day-old animals measuring 600–625 μm were usually competent and were used for all injection experiments. Larvae that were smaller than 600 μm showed a lower percentage of metamorphosis in response to 5-HT or the natural inducer. Older, larger larvae (>700 μm) were not useful for injection experiments as a relatively high percentage of these animals metamorphosed spontaneously and in response to FIO (control) injections. This obscured the effects of 5-HT and other experimental compounds.

To facilitate access to the larval hemocoel, larval shells were decalcified by culturing the animals in a low pH and calcium-free artificial seawater bath overnight (Pires and Hadfield, 1993). Larvae were reacquainted to normal seawater (pH 7.9) using several changes of FIO. Prior to injection, larvae were embedded in a 1% wgt/vol solution of low-melting-point agarose (Type VII, Sigma Chemical Co.). Animals were then teased from the gel and pipetted into the experimental chamber. This procedure temporarily impeded ciliary beating and swimming activity so that the larvae lay on the bottom of the dish or swam very slowly and were easily injected. Larvae recovered from this treatment within several hours and swam slowly in the test chamber. Injected animals were less active than uninjected animals, but showed no increase in mortality over the period of most experiments.

Decalcified larvae were injected with a small volume (about 6 nl or about 10% of larval volume) of the test solution using a Picospritzer II (General Valve Corporation) (McCaman *et al.*, 1977). The injection site was approximately 50 μm dorsal to the statocyst, just under the epidermis. This site was just lateral to the brain and avoided direct injection into ganglionic neuropils (Fig. 1) (Lin, 1995). Glass micropipettes were pulled on a Flaming/Brown Model P-87 automatic puller (Sutter Instruments).

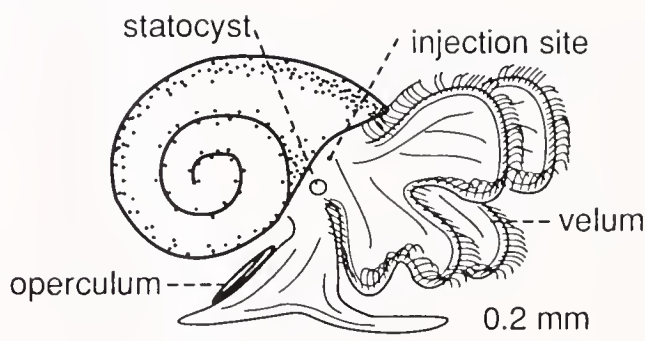


Figure 1. Diagram of a competent larva (redrawn from Fretter and Graham, 1962). Injection site is approximately 50 μm dorsal to the statocyst and probably adjacent to the cerebral ganglia (Lin, 1995).

Following injection, larvae were observed for signs of metamorphosis. Metamorphic induction was considered to have begun when ciliated cells were dropped from the larval swimming organ, or when the velum was dropped in pieces (Fig. 2). Results were scored at 48 hours; a positive result (metamorphosis) was scored only if metamorphosis was complete. Partial metamorphosis (animals retaining mounds of velar supportive cells or ciliated cells from the pre- or post-oral bands) (Pires and Hadfield, 1993) was not considered to be a positive result. Control and experimental animals were taken from the same culture. Positive (5-HT bath) (Levantine and Bonar, 1986) and negative (FIO bath) controls were included in each experiment to determine the capability of that age group for developmental change.

Test solutions

Fluoxetine was a generous gift from the Eli Lilly Research Laboratory; $\alpha\text{m}5\text{HT}$ and 5-HT were purchased from Research Biochemicals International; GABA, gramine, and L-ascorbic acid were purchased from Sigma Chemical Co. Chemical solutions used for injections were 10^{-4} M (unless otherwise noted) because larval response was usually optimal at this concentration. The optimal response for fluoxetine was seen at 10^{-6} M. Chemicals were introduced into solution in 1 ml of distilled, deionized water, and then diluted in 99 ml of FIO to achieve a 10^{-4} M solution. Solutions of 10^{-6} and 10^{-8} M were made by serially diluting aliquots of the 10^{-4} M solution. The one exception to this procedure involved gramine, which was first dissolved in 1% ethyl alcohol. Injections of 1% ethanol into competent larvae resulted in no significant induction of metamorphosis.

Data were analyzed with 2-way or 3-way chi-square tests (Sprinthall, 1994). For cells with low expected frequencies, a Yates correction was performed to ensure that the chi-square was not inflated (Sprinthall, 1994). Percentage data were normalized using an arc-sin trans-

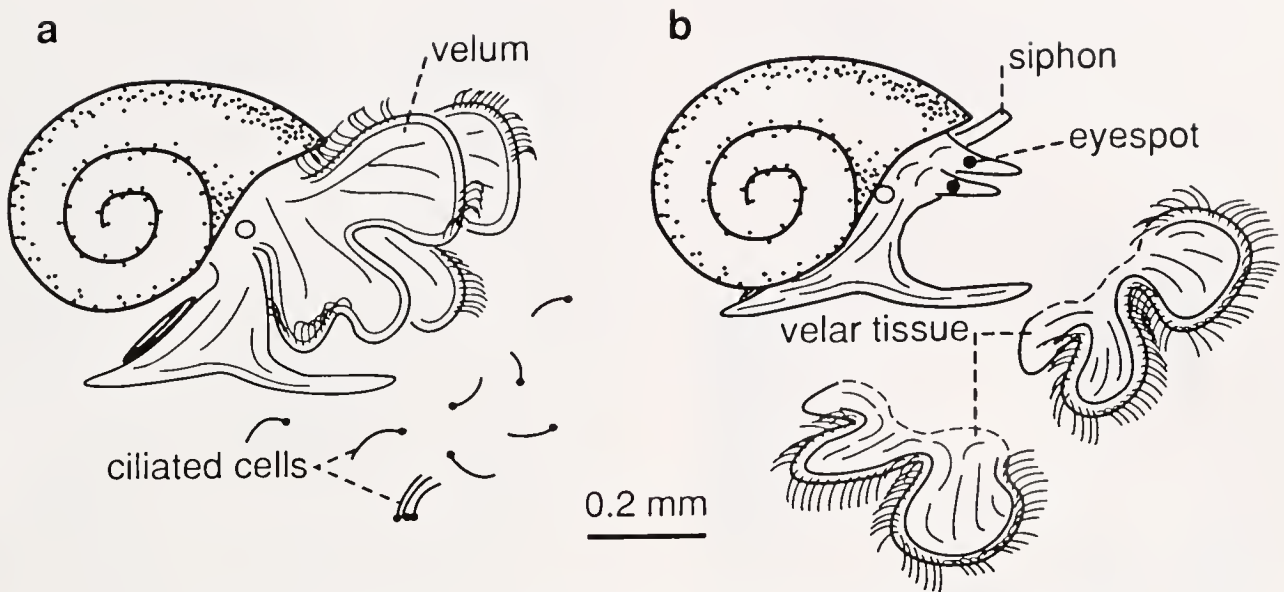


Figure 2. (a) Diagram of a larva at the beginning of metamorphosis. Ciliated cells are being individually dropped from the velum. (b) Drawing of a newly metamorphosed juvenile. The velum has been lost in large pieces (modified from Fretter and Graham, 1962).

formation before analysis (Rohlf and Sokal, 1981), but were transformed back to percentages for graphing. Error bars are standard deviations of the mean. Graphs were produced with the Statgraphics software program (Manugistics, Inc.).

Results

Injection and bath-application of 5-HT induced significantly more metamorphosis than did FIO controls (Fig. 3). The results of 5-HT injections were also statistically different from those produced by bath-application of 5-HT. Fluoxetine, a 5-HT reuptake inhibitor (Kandel, 1991; Frazer and Hensler, 1994; Ramamoorthy *et al.*, 1993), and α m5-HT, a 5-HT agonist (Walker, 1986; Chaouche-Teyara *et al.*, 1993), also induced significant levels of metamorphosis compared to controls (Figs. 4, 5). The results of 10^{-6} M fluoxetine injections did not differ statistically from those seen in the 5-HT bath condition. Injections of 10^{-4} M fluoxetine induced less metamorphosis than injections of 10^{-6} M fluoxetine (*cf.* Figs. 4, 6). When 10^{-4} M fluoxetine and 5-HT are injected together there is an additive effect (Fig. 6). Gramine, a 5-HT antagonist (Zhang and Harris-Warrick, 1994), did not promote metamorphosis when injected alone, and when injected along with 5-HT it resulted in decreased rates of metamorphosis (Fig. 7). To determine if these results were indeed specific to serotonergic neurons and their influence on larval physiology, GABA was injected as a negative control. Injections of this neurotransmitter did not induce significant levels of metamorphosis (Fig. 8).

5-HT Injections Induce Metamorphosis

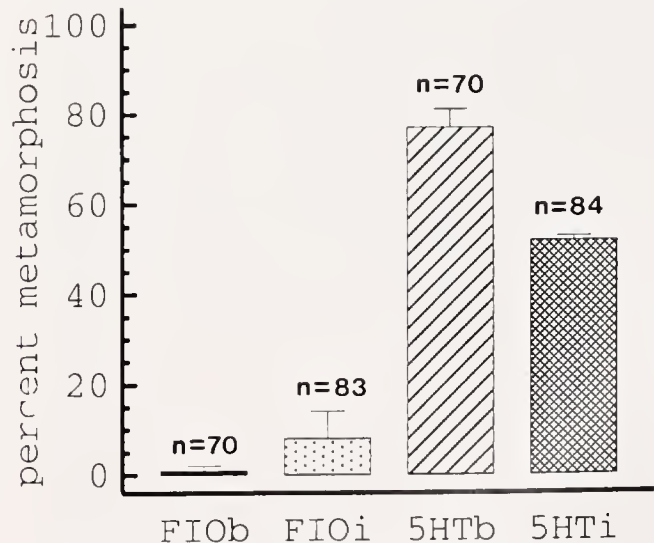


Figure 3. Injections of 10^{-4} M 5-HT (5HTi) induced metamorphosis in about 50% of larvae in comparison to FIO injections (FIOi) which induced an average of 8% metamorphosis ($\chi^2_{0.01(1)} = 31.69$). Larvae were scored after 48 hours in all experiments. Comparison of 5-HT bath (5HTb) and 5HTi showed a statistically significant difference between these two treatments ($\chi^2_{0.01(1)} = 11.02$). Control conditions using bath-applied 5-HT (5HTb) and bath FIO (FIOb) induced 77% and 1% metamorphosis, respectively.

Fluoxetine Induces Metamorphosis

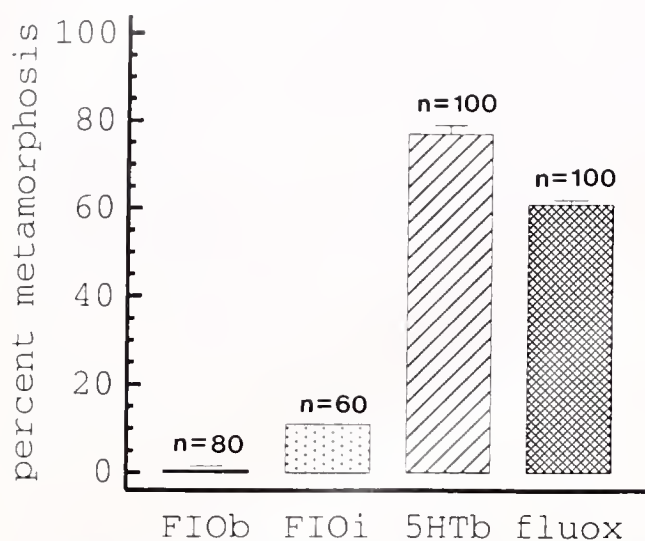


Figure 4. 10^{-6} M fluoxetine injections (fluox) induced 61% metamorphosis. Statistical analysis comparing 10^{-6} M fluoxetine injections with FIO injections showed a highly significant effect ($\chi^2_{0.01} = 34.67$). The fluoxetine treatment was not significantly different from the 5HTb condition ($\chi^2 = 2.42$; accept H_0).

Discussion

To partially test the hypothesis that serotonin and serotonergic neurons are directly involved in metamorphosis, behaviorally relevant amounts of 5-HT were injected into competent *Ilyanassa* larvae. Injections induced a high level of metamorphosis, suggesting that serotonin may be normally released as a neurotransmitter during metamorphosis. Because metamorphosis was considered to have occurred only if the loss of the velum and development to the juvenile state were completed within the experimental period, percentages of metamorphosis reported in this study may be somewhat less than those of other authors. Even with these more rigorous criteria, the results of our injection experiments were nonetheless significant.

Many compounds can be oxidized by seawater, losing or changing potency and thus acting in unexpected ways within larvae (Pires and Hadfield, 1991). Metamorphosis in response to bath-applied 5-HT could reflect the activity of this neurotransmitter within the larval nervous system or the activity of some unidentified oxidized product of this compound. Direct injection of 5-HT into the hemolymph eliminates the confounding factor inherent in bath-application studies, although it does not eliminate the possibility that serotonin is metabolized within larvae and that it is some metabolite, rather than serotonin, that was active during our experiments. Still, these

experiments allow us to further define the locus of 5-HT activity in the larval nervous system.

5-HT plays numerous roles in molluscan nervous systems (Walker, 1986) and major groups of serotonergic neurons have been identified in several gastropod taxa (Koshtoyants *et al.*, 1961; Walker 1986; Jahan-Parwar *et al.*, 1987; Goldberg and Kater, 1989; Diefenbach and Goldberg, 1990; McPherson and Blankenship, 1991; Fitzgerald and Carew, 1991). For example, 5-HT regulates neurite extension and growth-cone motility in the freshwater snail *Helisoma trivolvis* (Price and Goldberg, 1993); stimulates motor neurons in *Aplysia californica* (Ram *et al.*, 1991); plays an important role in molluscan peripheral tissues, especially the heart (Walker, 1986); and heightens motor activity in embryonic gastropods in a developmentally sensitive manner (Koshtoyants *et al.*, 1961; Diefenbach *et al.*, 1991). In these young animals exogenous serotonin increases ciliary activity during early embryonic stages, then sensitivity to 5-HT decreases to an intermediate level. Eventually, ciliary beating alternates with long pauses and contraction of the velar lobes (Koshtoyants *et al.*, 1961). Results from these studies suggest that 5-HT is an endogenous factor partially responsible for controlling the characteristic ciliary beating that occurs during embryonic development in many gastropods.

Full metamorphosis in the prosobranch *Ilyanassa* is similar to the partial metamorphosis of *Phestilla sibogae* seen in response to hydrogen peroxide and catecholes

Alpha-Methyl-5-HT Induces Metamorphosis

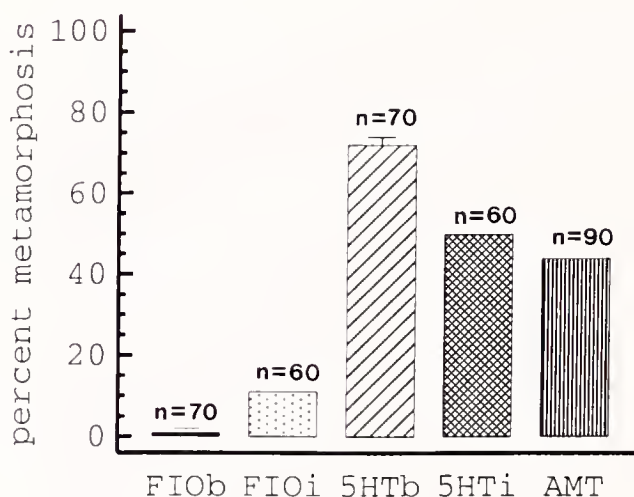


Figure 5. Injections of alpha-methyl-5-HT (AMT) induced 44% metamorphosis. This 5-HT agonist was as effective as 5-HT. FIOi induced 11% metamorphosis in this set of experiments. Metamorphic induction was significantly higher for AMT than for FIOi animals ($\chi^2_{0.01} = 17.97$).

Fluoxetine and 5-HT have an Additive Effect

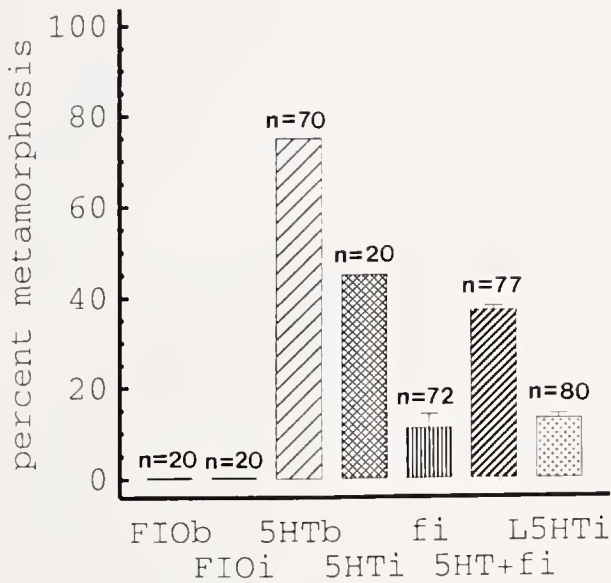


Figure 6. When 10^{-8} M 5-HT (L5HTi) and 10^{-4} M fluoxetine (fi) are injected in combination (5HT + fi) there is an additive effect in comparison to injections of these compounds alone ($\chi^2_{.01(2)} = 23.16$). 10^{-8} M 5-HT injections resulted in significantly less metamorphosis than injections of 10^{-4} M 5-HT ($\chi^2_{.01(2)} = 9.76$).

GABA does not Induce Metamorphosis

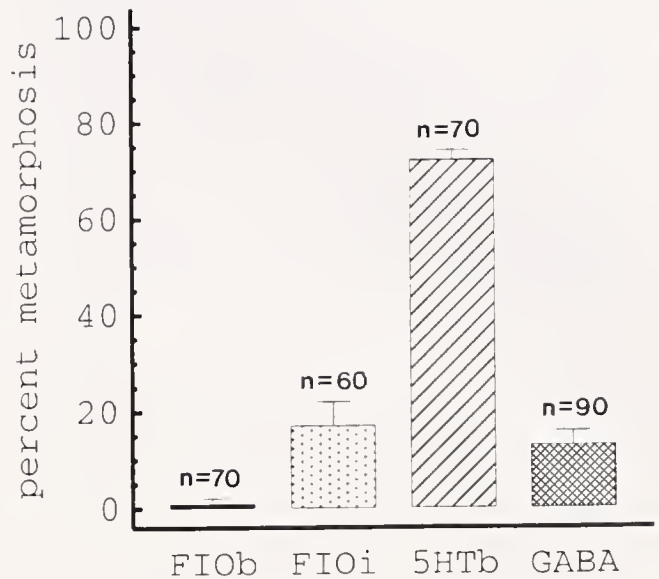


Figure 8. The amino acid GABA was used as a negative control. Results of GABA injections showed no increase in metamorphosis in comparison to FIOi controls, suggesting that metamorphosis is specific to 5-HT or 5-HT analog ($\chi^2 = 0.24$; accept H_0).

Gramine Inhibits 5-HT-Induced Metamorphosis

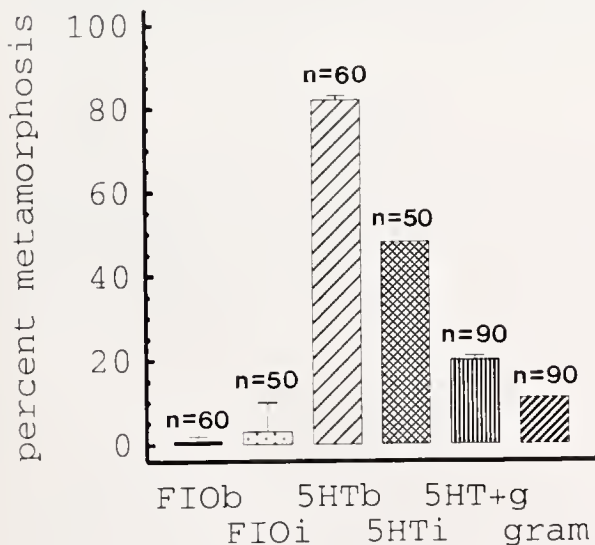


Figure 7. The 5-HT antagonist gramine reduces 5-HT-induced metamorphosis from 48% to 20%. A 3-way chi-square analysis comparing levels of metamorphosis induced by injections of gramine (gram), gramine + 5-HT (5HT+g), and 5-HT alone, showed that this reduction is significant ($\chi^2_{.01(2)} = 26.27$).

(Pires and Hadfield, 1991). The first sign of metamorphosis is the loss of velar metachronal rhythm (Bonar, 1973). This loss may be due to the uncoupling of gap junctions at the bases of the preoral ciliated cells. If the velar ciliated cells are separated from the central nervous system (CNS), coordinated ciliary arrests are abolished and the cilia show continuous metachronal beating (Mackie *et al.*, 1976). Studies by Koshtoyants *et al.* (1961) revealed the cilio-excitatory role of 5-HT, but electrophysiological studies have shown that spikes generated by electrical stimulation of the velar cells elicit ciliary arrests (Mackie *et al.*, 1976; Arkett, 1988), indicating that 5-HT is not responsible for cessation of ciliary beating. Serotonin is thus unlikely to be the neurotransmitter at neurociliary junctions but may act at extrasynaptic receptors (Mackie *et al.*, 1976). In *Ilyanassa*, 5-HT injections induce a high percentage of metamorphosis within 48 hours, but we do not know if the larval response to this injection involves a cilio-excitatory action of 5-HT, a modulatory effect on extra-synaptic receptors near the ciliated epithelium (Mackie *et al.*, 1976), activity on a separate set of cells—perhaps within the CNS, or a combination of these effects.

Both *Ilyanassa* and *Phestilla* lose their vella early in metamorphosis. *Phestilla* ingests its velar ciliated cells and incorporates the remaining supportive cells into the cephalic epidermis (Bonar and Hadfield, 1974). *Ilya-*

nassa may lose the velum *in toto* (Scheltema, 1962), or ciliated cells may drop off individually or in clumps (Couper and Leise, 1994). Like *Phestilla*, *Ilyanassa* metamorphoses a number of hours after initial exposure to an inducer (Bonar and Hadfield, 1974; Levantine and Bonar, 1986). The reason for this long delay is unknown.

Endogenous 5-HT may activate targets postsynaptic to the chemosensory neurons that mediate a larva's response to the natural inducer. Injections of 5-HT may mediate either the settlement response, which includes the cessation of ciliary beating, or the metamorphic response (loss of the velum) or both. Irrespective of its causative role in metamorphosis, if 5-HT is active as a neurotransmitter or neuromodulator, then 5-HT and related pharmacological agents should affect the metamorphic process. We tested several relevant compounds in bath-application and injection experiments in an attempt to support the idea that 5-HT has an internal mode of action. The results support the hypothesis that serotonergic neurons need to be activated for metamorphosis to proceed.

The compound α m5-HT is a 5-HT₂ agonist in vertebrate nervous systems (Chaouche-Teyara *et al.*, 1993) and a 5-HT agonist in invertebrate nervous systems (Walker, 1986). Although receptor classification is not identical for molluscs and vertebrates, structure-activity studies using 5-HT and chemical analogs indicate that the preferred form of the ligand for activation of any particular 5-HT receptor is probably similar for different species (Walker, 1986). As was expected, α m5-HT induced rates of metamorphosis similar to those seen with 5-HT injections. These results support the idea that 5-HT, and not a metabolite, is active internally as a neurotransmitter in *Ilyanassa*.

Gramine selectively antagonizes activity generated by 5-HT application in the crab stomatogastric ganglion (Zhang and Harris-Warrick, 1994). When 10^{-4} M gramine was injected into *Ilyanassa* along with 10^{-4} M 5-HT, induction of metamorphosis was half of that seen with injections of 5-HT alone. This decreased level of metamorphosis indicates that gramine inhibited the action of 5-HT in *Ilyanassa* larvae. Whether gramine and 5-HT compete for the same binding site is unknown.

GABA is a common molluscan neurotransmitter (Osborne, 1971) and can have mixed excitatory and inhibitory effects (Kerkut and Walker, 1961). It induces metamorphosis in *Haliotis* by mimicking the actions of the natural inducer at external chemoreceptors (Trapido-Rosenthal and Morse, 1985, 1986a,b; Barlow, 1990; Wodicka and Morse, 1991). Injection of GABA provided a negative control for our study.

In summary, 5-HT was clearly the most powerful metamorphic inducer of the compounds tested.

The synaptic effects of 5-HT are terminated by the binding of its molecules to specific transporter proteins

(re-uptake) in serotonergic terminals; this mechanism is prevented by selective 5-HT reuptake inhibitors like fluoxetine, which are used as effective antidepressants for humans (Kandel, 1991; Ramamoorthy *et al.*, 1993; Frazer and Hensler, 1994; Barondes, 1994). Fluoxetine acts to increase the availability of 5-HT at the synapse in a number of mammalian nervous systems (Sprouse *et al.*, 1993; Biegon *et al.*, 1993), although Sloley *et al.* (1993) provided evidence that this effect may not occur in all vertebrates. Uptake mechanisms for 5-HT occur in brains of the snail *Helix aspersa* and the squid *Loligo pealeii* (Osborne *et al.*, 1975; Feldman and Dowdall, 1973) and are probably widespread in molluscs. As anticipated, fluoxetine injections induced levels of metamorphosis similar to those of 5-HT injections. Fluoxetine injections also induced levels of metamorphosis that were not statistically different from those seen in 5-HT bath conditions. However, 5-HT injections produced rates of metamorphosis that were statistically different from 5-HT bath-application rates. Injected 5-HT may not be available for an extended time in the nervous system of *Ilyanassa*. Injecting a 5-HT reuptake inhibitor could allow 5-HT to persist in the larval nervous system for a longer time, enabling it to exert its metamorphic effects to a greater extent.

The additive effect seen with injections combining 10^{-8} M 5-HT (which by itself induces low levels of metamorphosis) and 10^{-4} M fluoxetine corroborates that serotonin and serotonergic neurons are involved in metamorphosis. It is unclear whether 5-HT acts in the nervous system of *Ilyanassa obsoleta* larvae as a CNS neurotransmitter or as a neuromodulator, but the results of this study provide evidence that it is an endogenous compound that plays an important role in metamorphosis.

Acknowledgments

We thank Drs. Gilbert Gottlieb and Cheryl Logan for reviewing this manuscript. Some of the data presented here were previously described in abstract form (Couper and Leise, 1994). We also thank Drs. Anthony Pires and Daniel Rittschof for providing emergency algal cultures and egg capsules.

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Antennal Responses to Hydrodynamic and Tactile Stimuli in the Spiny Lobster *Panulirus argus*

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Abstract. The responses of the long, spiny, antennal flagella of the spiny lobster *Panulirus argus* to hydrodynamic and tactile stimuli were investigated. Experiments were performed in the dark and included videographic laboratory studies of small tethered lobsters (<20 mm carapace length) and nighttime field observations of larger, subadult, foraging animals. The antennae are held laterally in both tethered and free-ranging animals. Water jets trigger bilateral antennal responses in which both flagella are swept forward for rostrally directed stimuli, backward for caudal stimuli, and in an intermediate backward direction when stimulated laterally. Mean response angles are greater for caudal stimuli (17°–48°) than for rostral stimuli (10°–16°), and lobsters exhibit lateralized sensitivity when jets are directed from the caudal sector, as indicated by larger ipsilateral responses—up to twice the amplitude of contralateral responses in field experiments. Untethered lobsters frequently turn the body in the direction of the water jet and tailflip away or tailflip without first turning. Tactile stimuli to the lateral edges of the antenna, carapace, walking leg, abdomen, and tailfan also trigger primarily backward sweeps of the antennae. Only the antennule and medial antennal receptive fields yield forward movements, and these elicit smaller responses (mean response $\leq 5^\circ$) than in the backward direction (mean responses up to 15°). Threshold tactile stimuli trigger exclusively ipsilateral responses; thus, lateralization is absolute. These results demonstrate that spiny lobsters accurately localize mechano-

sensory stimuli and direct their antennal flagella in the perceived direction, a response consistent with a defensive function of the antennae in these nonchelate decapods. Overall sensitivity is greatest for hydrodynamic stimuli, a result interpreted as being important for the detection of and defense against large predatory fish whose nearby movements would generate broad, directional, water-current pulses.

Introduction

The crustacean second antenna is a prominent sensory appendage among aquatic arthropods, often terminating in a long segmented flagellum. A variety of setae and sensilla are found both on the proximal segments and along the annuli of the flagellum. These structures are believed to convey mechanosensory as well as chemosensory information (Derby, 1982), as do similar hairlike structures located elsewhere on the cephalic and thoracic appendages. The antennae are also used to signal behavioral information, for example during aggression (Bovbjerg, 1956). A specialized “antenna waving” behavior has been described in crayfish (Rubenstein and Hazlett, 1974) and in the clawed lobster *Homarus* (Solon and Cobb, 1980). In the crayfish, antennal waving signals appeasement following agonistic encounters (Ameyaw-Akumfi, 1979) and, although clearly functioning as a visual stimulus, also transmits tactile information (Bruski and Dunham, 1990). In *Homarus* the antennal flagellum contacts the claws of conspecifics and is perceived as a mechanosensory stimulus.

In the spiny lobster the antennae assume additional functions in defense, activities performed by the chelipeds in most other decapods. The Caribbean spiny lob-

Received 3 July 1995; accepted 29 April 1996.

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ster *Panulirus argus* uses the antennae additionally to make contact with other lobsters in the formation of migratory queues (Herrnkind, 1969). Unlike those of other crustaceans, the antennal flagellum in the spiny lobster is a robust appendage, well armed with forward-projecting spines similar to those on the carapace and other appendages. Pointed toward a potential predator, the antennae constitute a formidable defensive perimeter, particularly when many animals occupy a den, as is common in *P. argus* (Kanciruk, 1980). In fact, the antennae are used actively to fend off predators by directing the flagella toward the predator. If contact is made they push vigorously with the flagella to prevent further approach. If grasped or twisted, the antennal flagellum is often autotomized and a new, well-formed flagellum is regenerated at the next molt. The flagella are also brandished vigorously during agonistic encounters between conspecifics (Atema and Cobb, 1980).

For the spiny lobster, vision is clearly an important sensory modality for coordinating defensive antennal movements. Casual observation in the field or laboratory notes that the antennae respond readily to motion, even when the objects are moving above the water surface and therefore providing no mechanical cues. For example, animals in a laboratory tank typically rotate their antennae backward over their carapace in response to overhead movements. Slight changes in background illumination, even under dim light, cause lobsters to elevate their antennae to a more "alert" position. However, lobsters and other animals must rely increasingly on nonvisual sensory input during the nighttime hours, a period when lobsters are most active. Accordingly, the present study was initiated to test the antennal behavior of lobsters to nonvisual mechanosensory signals.

Mechanosensitivity in the spiny lobster antenna has been the subject of numerous studies (Laverack, 1964; Hartman and Austin, 1972; Tazaki and Ohnishi, 1974; Vedel and Clarac, 1976; Vedel, 1985), as has the reflex control of the antenna based on both local and distributed (leg) proprioceptive feedback (Schöne *et al.*, 1976; Vedel, 1980; Neil *et al.*, 1982). However, there has been no systematic investigation of antennal behavior in response to mechanosensory stimulation in the spiny lobster. In contrast, crayfish antennal behavior has been examined under a variety of conditions, *e.g.*, in response to controlled experimental stimuli such as water vibrations and water jets (Tautz, 1987; Schmitz, 1992), to natural hydrodynamic stimuli produced by small fish (Breithaupt *et al.*, 1995), and to tactile stimulation of the animal (Sandeman and Wilkens, 1983; Tautz, 1987; Sandeman, 1989). Antennal behaviors in the crayfish range from exploratory to stimulus tracking, and their role in prey localization has now been established (Sandeman and Varju, 1988; Zeil *et al.*, 1985; Breithaupt *et al.*, 1995).

In the present study we examine antennal behavior in the spiny lobster *Panulirus argus* in response to tactile and hydrodynamic (water jet) stimulation under controlled laboratory conditions. In addition, we observed the behavior of free-ranging lobsters in the field in response to the same type stimuli. Touch and sudden, localized water currents are stimuli representative of the environmental signals naturally encountered by lobsters while foraging at night in the open environment—for example, those produced by sharks, sea turtles, other large predatory fish—and by structural components of the environment including macroalgae, sea grasses, gorgonians, etc. Previous studies have demonstrated that lobsters utilize the hydrodynamic signals associated with waves and currents for orientation and locomotory behaviors (Walton and Herrnkind, 1977; Nevitt *et al.*, 1995). In the following experiments, we address the question of nonvisual mechanosensitivity and whether this type of information alone is effective in coordinating antennal movements in defensive behaviors. Our results indicate that lobsters direct their antennae toward the source or location of the stimulus, and that they distinguish directional signals from the front, from behind, or from alongside the animal.

Materials and Methods

Laboratory studies

Observations and experiments were conducted on juvenile spiny lobsters collected in the Florida Keys and shipped to the Florida State University Marine Laboratory, Turkey Point, Florida, and to the University of Missouri—St. Louis. The animals ranged in size from 12 to 19 mm carapace length and were housed either in unfiltered running seawater or artificial seawater and were fed cut squid and shrimp. Experimental results were based on responses from eight animals. Lobsters were individually marked for identification.

Antennal responses were recorded from animals tethered in the center of a 30 × 30 × 7 cm chamber filled with fresh seawater. To achieve near-normal posture and freedom of movement of the appendages during the experiment, a flexible, lightweight tether (1-kg test weight fishing line) was attached ventrally to the triangular sternum at the base of the legs and threaded through a small pivot hole in the bottom of the chamber. The tether allowed the animals freedom to turn and walk in place on the slick Plexiglas surface of the chamber floor. The chamber was centered on a turntable and rotated by hand.

The mechanosensory receptors of the lobster were stimulated by touch and by brief water-current pulses. For tactile stimulation a thin nylon filament (0.2 mm diameter, 7 cm long) attached to a thin wooden stick was

manipulated by hand to lightly stroke the surface of the animal. This type of stimulus characteristically triggered ipsilateral antennal movements, although in some cases overt behaviors were not observed. Preliminary tests using a No. 4 camel-hair brush induced more pronounced responses including bilateral antennal movements, turning, and locomotion. The filament-stimulus technique was adopted because the resulting threshold responses were more site specific. Also, with the larger brush it was difficult to localize the stimulus when small animals were used or to rule out stimulation by near-field water currents prior to contact. Tactile stimuli were delivered randomly at different locations at 2-min intervals by drawing the tip of the filament in a single stroke over the following receptive fields (Fig. 1): lateral margin of the antennular flagellum (antennule), medial and lateral margins of the antennal flagellum (*i.e.*, front and back edges of the second antenna as seen in the resting posture; antenna med, antenna lat), lateral surface of the carapace (carapace), dorsal surface of the fourth leg (walking leg), lateral margins of the abdominal tergites (abdomen), and dorsal surface of the endopod and exopod (tailfan). These experiments were conducted in dim light and with the eyes of the lobster coated with black acrylic

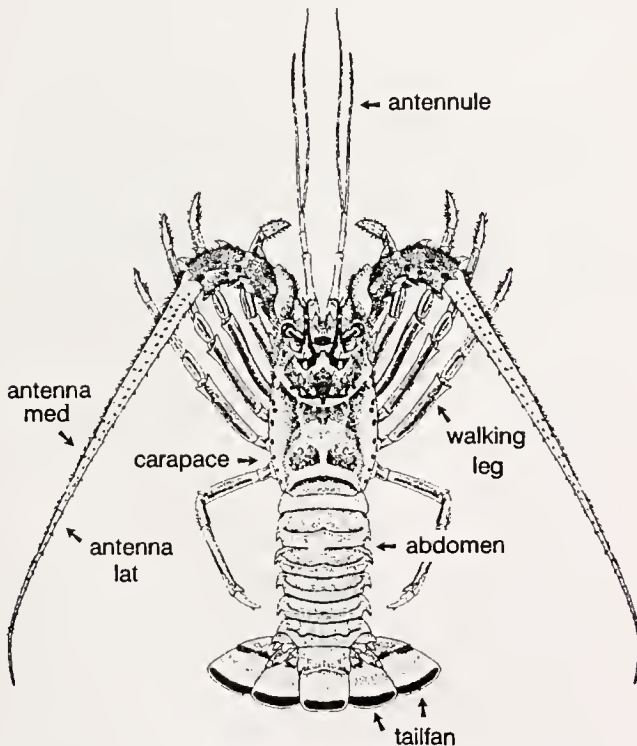


Figure 1. The spiny lobster *Panulirus argus* showing locations of tactile stimuli: antennule, medial antenna, lateral antenna, carapace, walking leg, abdomen, tailfan (endopod and exopod). Drawing of lobster adapted with permission from Sterrer (1986), Fig. 108.

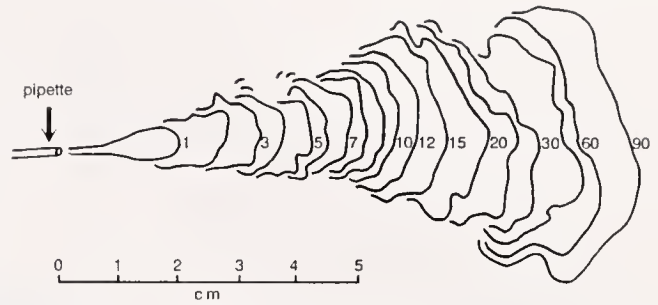


Figure 2. Laboratory water-jet stimulus visualized by addition of dye to the seawater-filled pipette. Numbered traces are video frames (30^{-1} s) and illustrate a water-jet stimulus pulse over a 3-s interval.

paint to further reduce visual stimulation. Responses attributed to visual stimuli were observed only if the eyes were shadowed directly; the response was presumably due to changes in light intensity but not movement *per se*. The paint was usually cleaned off overnight by the lobster using its pereopod tips.

Water-current stimulation was produced by jets of water delivered from the tip of a disposable pipette directed toward the center of the test chamber. The pipette tip was positioned 5 cm from the center of the chamber and 2.5 cm above the chamber floor and was pointed downward at an angle 15° from horizontal. Preliminary tests showed this to be the most effective position on the basis of the strength of the stimulus jet and the size of the animals. A constant water-jet stimulus was delivered by depressing the rubber bulb of the pipette with an electro-mechanical transducer (Pasco Vibrator, Model SF-9324). The transducer was driven by pulses from a function generator (Tektronix FG 501) fed through a power amplifier and triggered by a stimulator (Grass S5). The stimulus pulse was calibrated by single-frame analysis of videotaped test pulses produced with a dye-filled pipette (Fig. 2). Mean axial velocity of the stimulus pulse at 5 cm, the distance to the center of the chamber, was $11.7 \text{ cm/s} \pm 4.1 \text{ cm/s SD}$ ($n = 7$). Stimulus intensity, therefore, is within the range (4–20 cm/s) of suprathreshold pulse intensities that reliably elicit antennal responses in crayfish (Schmitz, 1992), but somewhat higher than threshold values (0.1–6.7 cm/s) for crayfish turning and escape responses (Ebina and Wiese, 1984). Overlays of the test-pulse image reveal that most of the dorsal body surface, excepting about the distal half of the antennae, is enveloped by the water jet.

Stimuli were presented from various angles, depending on the position of the turntable, the animal's turning movements, or both, and were presented at intervals of at least 2 min followed by rotation of the chamber. Stimulus jets were delivered only when the lobster was stationary with its abdomen fully extended and with both

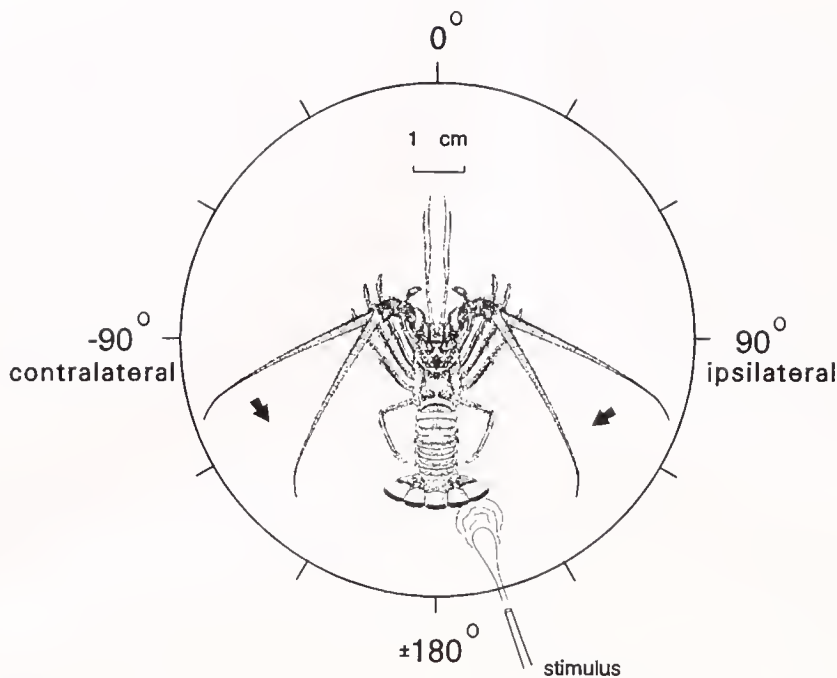


Figure 3. Stimulus and response angles for water-jet hydrodynamic stimulation. Stimulus angles are referenced to the middle of the carapace. A 0° stimulus is from in front of the lobster, $\pm 180^\circ$ is from behind; positive values denote ipsilateral stimulation. A stimulus is illustrated at $+165^\circ$. Angular antennal movements were measured with reference to the lobster's midline at the base of the antennal segments. Antennal end positions were subtracted from start positions so that sweeps of the left and right antenna (SLA, SRA) are denoted by negative values for backward rotation and positive values for forward rotation. Calibration scale corresponds to laboratory experiments with small animals.

antennae in the resting position, more-or-less perpendicular to the body axis. The water-jet experiments were conducted in the dark using infrared illumination (Sylvania, Mini-Minor) and an IR-sensitive CCTV camera (Baxall, CDG) to monitor antennal responses. Antennal behavior was recorded using a videocassette recorder (JVC Model VC-378U) and measured directly from the video monitor during playback. Rotation of the flagellum was measured with reference to a point just anterior to the basal segments of the antennae; stimulus angle was referenced to the middle of the carapace (see Fig. 3).

Field studies

To complement the laboratory experiments, antennal responses were elicited from lobsters foraging or walking normally in their natural habitat. Our purpose was to determine whether lobsters exhibited similar responses to roughly analogous stimuli while in the open at night and vulnerable to predators. Observations were made while skin diving over areas of vegetated hard-bottom substrate at depths of 1–2 m near the Keys Marine Laboratory, Long Key, Florida. Animals whose carapace length was between about 50 and 70 mm were tested by

stimulation devices scaled up to the larger juvenile and subadult-sized animals. For tactile stimulation a thin, flexible plastic rod (25 cm long, 3 mm in diameter) attached to a hand-held lobster "tickle stick" was used to stroke the animal. Water jets were delivered *via* a hand-operated kitchen baster extended by the addition of a 1-m length of PVC pipe (2.5-cm diameter O.D.). A thin plastic rod was attached to the tip so that when positioned above the middle of the animal it served as a gauge for presenting water jets at a constant distance. We confirmed that the jets made contact by observing the disturbance of fine sediment in front of the gauge. A small cloud of sediment was clearly visible below the stimulated lobsters.

Antennal behavior was tested as the lobsters foraged at night in the open so that the animal could be stroked or squirted by seawater at various sites or directions. On the nights of observation (23–26 June 1994), moonrise ranged from 1951–2219 h EDT, so natural moonlight was low or absent during the dive periods (~ 2100 – 2400 h). Observations were made using dive lights equipped with red filters ($\lambda = 680$ nm) to minimize visual disturbance of the animals. Previous experience indicated that lobsters are insensitive to red light, and under these conditions they continued foraging undis-

turbed. One diver presented the stimulus while a second diver recorded antennal responses. Diver training sessions were conducted to standardize presentation of stimuli and recording of antennal responses. Antennal positions were noted prior to the stimulus and marked on data sheets constructed on nontear paper (Avery #6725) predrawn with lobster silhouettes and 60° sectors of possible antennal rotation. After stimulus presentation, approximate final positions were noted along with stimulus location and direction and the estimated size of the lobster. Each animal was stimulated no more than twice, with lobsters selected as they were encountered. Only stationary lobsters were tested, as in the laboratory experiments; preliminary trials indicated that walking animals were less responsive and that accurate estimation of response angles was made more difficult.

Data analysis

The parameters for measuring stimulus and response angles are diagrammed in Figure 3. A water jet stimulus of 0° is from the front of the lobster and $\pm 180^\circ$ is from behind; positive angles represent ipsilateral stimuli and negative angles contralateral. Start positions (resting) and end positions ranging from 0° to 180° and sweep angles (*i.e.*, start minus end positions such that backwards sweeps are negative in sign and *vice versa*) were evaluated for both antennae (see Fig. 3). As in a previous study in the crayfish *Procambarus clarkii* (Schmitz, 1992), responses of the left and right antenna did not differ significantly. Thus, data have been pooled and means and standard deviations, 95% confidence intervals, and circular means and angular deviations (Batschelet, 1981) are evaluated for the antennal responses. Significant differences are based on 95% confidence intervals. Circular means are not shown here because they do not differ by more than 2.4° (usually by about 0.1°) from the linear means.

Results

Antennal responses to hydrodynamic stimulation

Laboratory studies. Animals tethered in the experimental chamber were allowed to adapt for 30 min. Occasionally animals walked or tailflipped spontaneously during the experiments. However, tailflips were never elicited by mechanical stimuli. Quiescent lobsters frequently exhibited a slow metachronal stepping rhythm, an activity pattern observed in other decapods, and rested with the abdomen extended and the antennae pointing laterally at a mean angle of 90.5° (see Fig. 4, “+” symbols). For experimental purposes, stimuli were delivered only when the antennae pointed laterally. In walking animals the abdomen was also extended, but antennal positions were somewhat more varied and occa-

sionally the flagellum was actively swept forward and backward.

Whether quiescent or walking, animals generally held the antennal flagellum at an upward angle of about 10°–15°. Infrequently, the antennae were raised higher, as estimated by the shadow cast from the IR source. The more horizontal antennal posture differs from that of quiescent animals in lighted holding tanks; under those conditions the flagellum often points vertically. A vertical antennal position may be more characteristic of unrestrained animals that always seek a corner of the tank or natural structure with vertical relief, *e.g.*, a sponge; during experiments, animals were tethered in an open unstructured environment. The antennal posture of larger lobsters encountered while they are actively foraging is similar to that observed in the tethered animals. Unless disturbed, the antennae point laterally, whether the animal is walking or stationary during feeding, and the abdomen is always extended.

Water jets delivered to small tethered lobsters elicit a bilateral antennal response in which the flagella are swept forward or backward depending on the stimulus direction. No antennal response was observed in 12% of the stimulus trials ($n = 30$ of 252). Initial movement is rapid and occurs within 0.5–1.0 s; a secondary movement of the flagellum may follow at a slower rate. The initial, more consistent antennal response is presented in this study and is further distinguished from antennal movements associated with startle responses involving locomotion. Antennal start and end positions (mean and 95% confidence intervals from eight experiments) relative to the stimulus angle are illustrated in Figure 4. Mean start positions for different stimulus angles range between 82.9° and 99.5° and average $90.5^\circ \pm 18.5^\circ$ SD, perpendicular to the rostrocaudal axis. Mean end positions reflect a general rotation of both antennae toward the stimulus, *i.e.*, sweeps in a rostral direction for frontal stimulation (0°–60°) and caudally for lateral or caudal stimulus angles (>60° to 180°; Fig. 4). Significant differences between antennal start and end positions for a given stimulus angle class are found only with caudal stimulation (>150°–180°) as indicated at $\pm 165^\circ$.

Thus, tethered juvenile spiny lobsters show bilateral antennal sweeps that are directed forward for frontal hydrodynamic stimuli and backward for all remaining stimulus directions. Sweep angles irrespective of antennal position are illustrated in Figure 5 as means for the 30°-stimulus angle classes. Significant differences demonstrate that antennal responses reflect the stimulus direction in the broad categories of frontal (0°–60°), lateral (>60°–150°), and caudal (>150°–180°); that is, backward sweeps are significantly greater for caudal *versus* lateral stimulation, and frontal stimuli produce forward sweeps

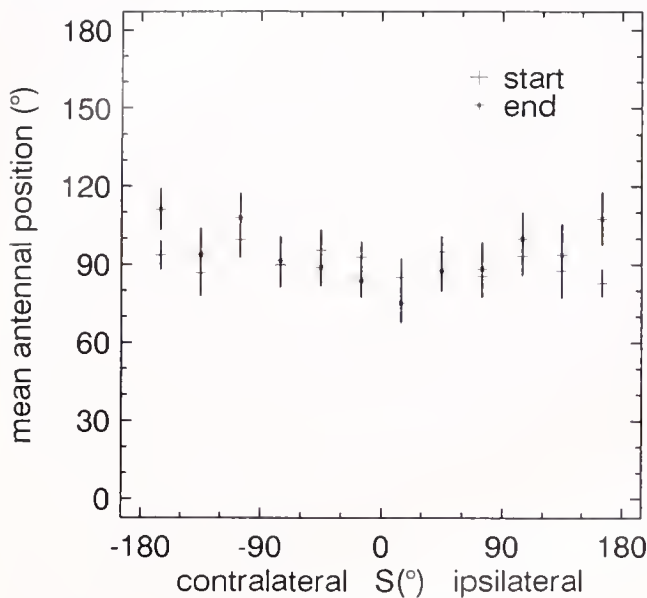


Figure 4. Antennal positions of small, juvenile spiny lobsters in laboratory experiments along with responses to ipsilateral (positive S -values) and contralateral (negative S -values) water-jet stimuli. Mean start (+) and end (*) positions are shown along with 95% confidence intervals ($n = 15$ – 29) for individual 30° stimulus categories; categories are represented graphically at their midpoints, e.g., 15° for 0° – 30° stimulus angles. See Figure 3 for illustration of measurement parameters.

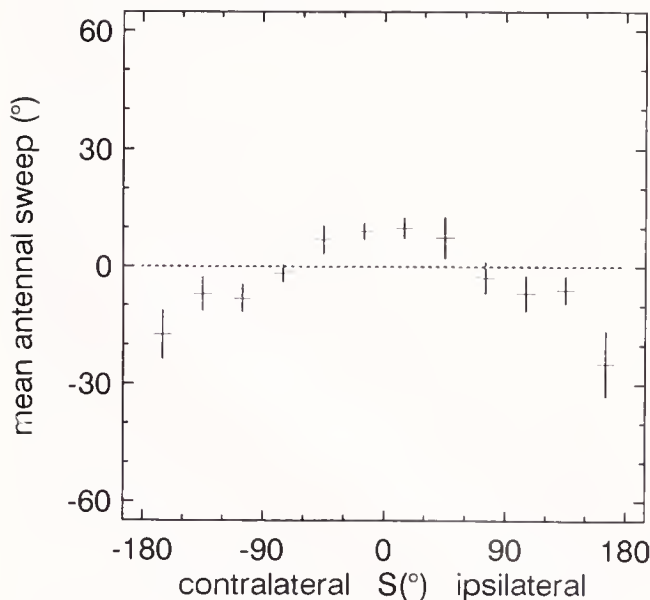


Figure 5. Dependence of antennal sweeps on the direction of water-jet stimuli in laboratory experiments with juvenile spiny lobsters. Responses are illustrated as means of antennal sweep amplitude with 95% confidence intervals for 30° stimulus angle classes. The antennal sweep data are based on the start and end positions shown in Figure 4.

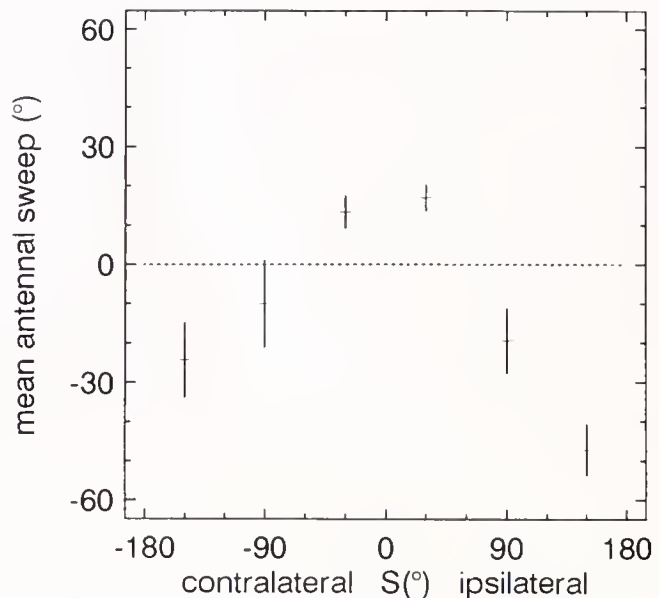


Figure 6. Dependence of antennal sweeps of foraging juvenile and subadult-sized spiny lobsters on the direction of a water-jet stimulus. Mean sweep amplitudes with 95% confidence intervals ($n = 7$ up to 33) are shown for 60° stimulus angle classes. Data are results of field experiments and compare with laboratory results in Figure 5.

that differ significantly from those of other directions. These results indicate that spiny lobsters not only distinguish between frontal and caudal stimuli, but also are able to more accurately localize the stimulus. However, responses for corresponding stimulus angles on the ipsilateral and contralateral side of the animal are not significantly different (Fig. 5), and indicate that the mean antennal response is statistically symmetrical. Although not significantly different, the mean ipsilateral response to caudal stimulation is greater by 7° and is consistent with a much greater ipsilateral response observed in the field studies (see below).

Field studies. Observations of 119 antennal responses were recorded in juvenile and subadult-sized spiny lobsters in their natural habitat, with lobsters receiving either one or two stimuli. Despite inherent variation in the strength of the hand-delivered stimuli, the lobsters responded in a consistently patterned way. Mean antennal sweeps in response to water jets are shown in Figure 6 and correspond closely to those analyzed in the laboratory (*cf.* Fig. 5). Again, frontal stimulation (0° – 60°) produces bilateral forward sweeps, whereas both antennae sweep backward for stimulus directions greater than 60° . Response amplitudes also increase as the stimulus shifts caudally. For stimuli in the caudal sector (150° stimulus-angle class) lobsters are able to lateralize, *i.e.*, to differentiate between water jets from the right and left side, as indicated by significantly greater antennal sweeps of the

ipsilateral antenna. Overall, sweeps from larger untethered animals in the field, especially those of the ipsilateral antenna, show nearly twice the amplitude of those from smaller tethered lobsters in laboratory experiments. The number of no-response trials was low (5%; 6 of 119).

Behavioral observations in the field revealed features of the lobster's response to hydrodynamic stimuli that were not observed in tethered animals. After the antennal response, lobsters frequently reacted without further stimulation—either turning toward the stimulus and then tailflipping away or tailflipping immediately (Table I). In approximately one-third of the stimulus trials involving lateral or caudal stimuli, lobsters turned toward the stimulus; frontal stimuli did not elicit turning. Turn angles were not measured, but in most instances the lobster rotated its body axis to face the approximate stimulus direction. The turning response, and its absence for frontal stimulation, is further indication of the lateralization in sensitivity to off-axis hydrodynamic stimuli. Tailflips occurred to a varying degree for all stimulus directions, but again at higher frequencies for more caudal directions. The escape response occurred both independent of ($n = 16$) and subsequent to ($n = 19$) the turning response.

Antennal responses to tactile stimuli

Laboratory studies. The antennal response elicited in small lobsters by touching the antennule, the medial or lateral edge of the second antenna, the carapace, a walking leg, the abdomen, or the tailfan is illustrated in Figure 7. The light strokes from a flexible nylon filament appear to be near threshold because responses were elicited only from the ipsilateral antenna: no response was observed in nearly one-third of the trials (31 of 108). The antenna is swept forward when contact is made with the antennule or the medial edge of the long antennal flagellum, although no response was observed in nearly half of the tests (45%, 10 of 22). All the remaining tactile stimuli elicit backward sweeps, and with a greater response frequency (76%); out of 86 total tests only a single forward sweep was observed. Thus, small lobsters move their long antennae toward the location of a tactile stimulus

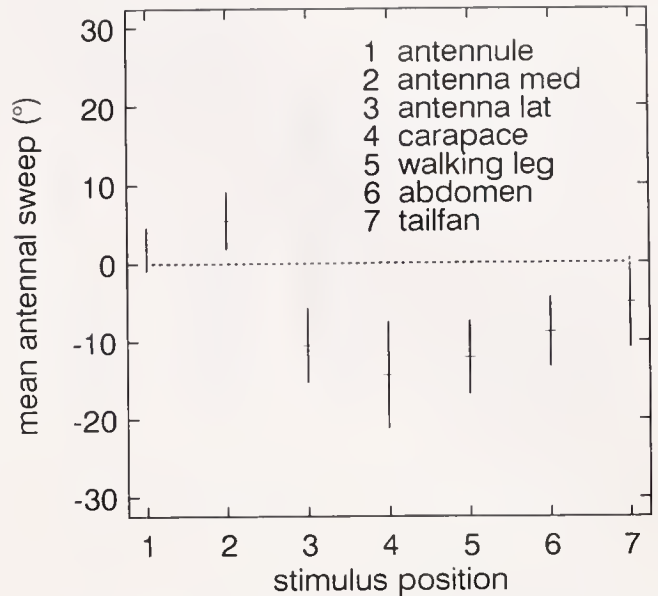


Figure 7. Dependence of sweeps of the ipsilateral antenna on the location of tactile stimuli in laboratory experiments with small juvenile spiny lobsters. Mean sweep amplitudes and 95% confidence intervals ($n = 7$ up to 20) are shown; stimulus locations are indicated in the inserted legend.

and accurately distinguish tactile sensory input from anterior and posterior body regions. Lateralization of stimulus sensitivity was absolute; only the ipsilateral antenna responded to stimulation either of the appendages or of the lateral surface of the carapace and abdomen. The distinction between rostral and caudal was especially pronounced for antennal stimulation—responses were significantly different and resulted in forward and backward sweeps when the stimulus was applied respectively to the medial and lateral edge of the flagellum. Overall, forward and backward responses differed significantly. However, within the categories of tactile stimuli that produce forward and backward responses, sweep amplitude is not significantly different (see Fig. 7).

Field studies. In their natural environment, juvenile and subadult-size lobsters responded to tactile stimuli (Fig. 8) in similar fashion to the smaller animals tested in the laboratory (*cf.* Fig. 7), except that responses were frequently bilateral, involving simultaneous sweeps of both antennae in more than two-thirds of the stimulus trials. The tactile stimulus applied under field conditions undoubtedly was more variable and presumably involved higher suprathreshold strengths. Due to the difficulty of observing both antennae and recording accurate response angles without aid of video playback, responses are not quantified by amplitude of antennal sweep. However, antennal responses in field-tested lobsters generally involved large sweeps ($>30^\circ$), and the an-

Table I

Turning and tailflip escape behaviors of foraging spiny lobsters in response to hydrodynamic water-jet stimuli

Stimulus sector	0°–60°	>60°–120°	>120°–180°
Turns (%)	0	36*	37
Tailflips (%)	14	31	54
Number of trials (n)	37	36	35

With one exception (*), all turns were ipsilateral or toward the direction of the stimulus.

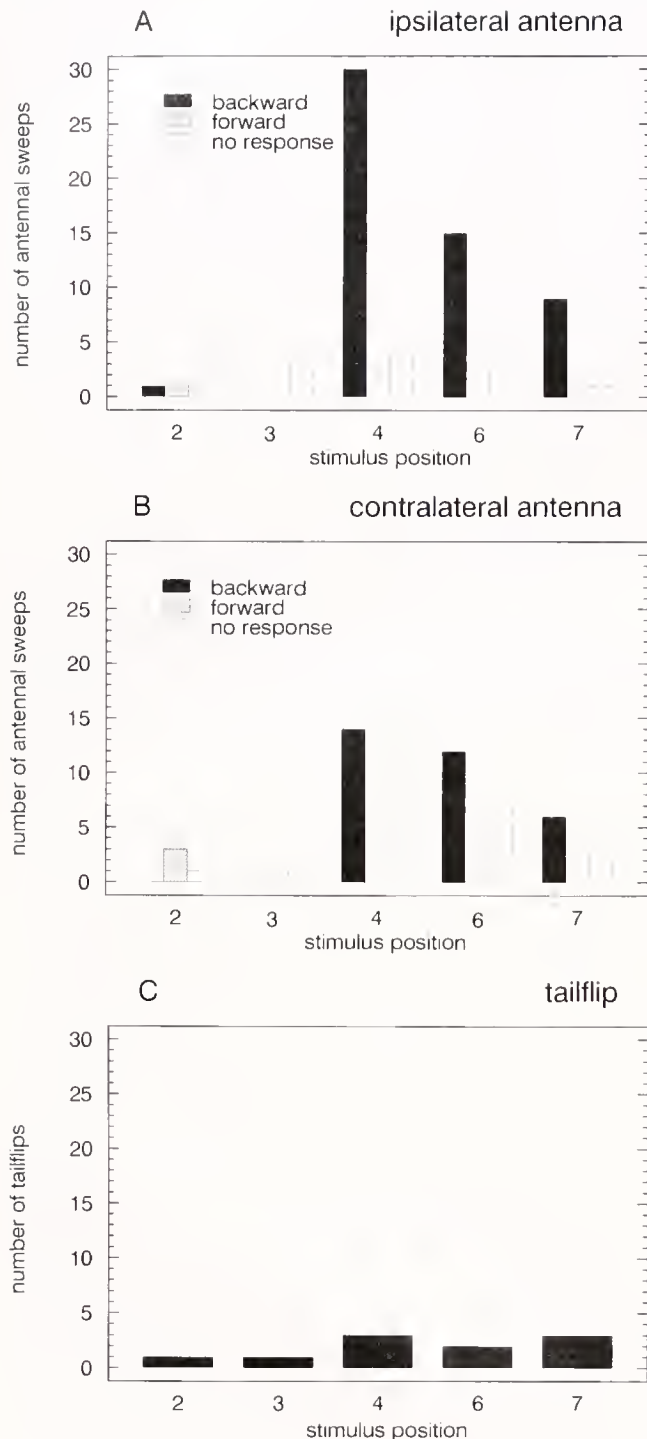


Figure 8. Antennal response frequencies to tactile stimulation in juvenile and subadult-sized spiny lobsters in field experiments. Ipsilateral (A) and contralateral (B) antennal responses are illustrated separately for the following stimulus locations: 2—antenna medial, 3—antenna lateral, 4—carapace, 6—abdomen, 7—tailfan, as in Figure 7. Bars represent backward sweeps (filled), forward sweeps (hatched), and no response (unfilled) of the antenna in both A and B. Tailflips elicited by corresponding tactile stimuli are illustrated in C.

tenna frequently crossed over the midline of the animal. In correspondence with the laboratory data, the ipsilateral antenna is swept backward when the lateral surface of either the carapace or abdomen, or the dorsal surface of the tailfan uropods, is touched (Fig. 8A); response failures were low (11%, 8 of 72). Similarly, the contralateral antenna responds vigorously with backward sweeps in response to carapace, abdomen, and tailfan stimulation (Fig. 8B), although the failure rate was higher (39%, 24 of 61). Forward sweeps of the antennae were never observed for thoracic or abdominal stimulation. Forward sweeps were elicited, however, by touching the medial edge of either antenna (stimulus position 2, Fig. 8A, B). Foraging lobsters did not respond to the few stimuli applied to the lateral edge of the antenna (stimulus position 3, Fig. 8A, B). Antennule and leg stimulation was omitted due to the uncertainty of localizing the stimulus to these appendages under field conditions. In addition to antennal movements, tailflipping behavior was elicited by tactile stimulation in field experiments, with tailflips occurring more often when more caudal surfaces of the animal were touched (Fig. 8C). Tailflip backward swimming rapidly steered the lobster away from the stimulus irrespective of the stimulus site.

Discussion

Mechanosensory-induced antennal behavior in the spiny lobster

We have analyzed the behavior of the spiny lobster *Palaeomonetes argus* in response to hydrodynamic and tactile stimuli. Quantitative laboratory studies on small juvenile lobsters and semiquantitative field analyses on larger animals under natural field conditions were designed to demonstrate mechanosensory functions independent of the visual system. The primary behavioral response is an immediate sweep of the antennal flagella in the direction of the stimulus. Laboratory and field results are consistent with regard to the antennal response, but related aspects of the behavior differ somewhat. For example, antennal movements were the sole overt response in laboratory experiments in which the strength of both water jet and tactile stimuli was closer to threshold and carefully controlled. Under field conditions the antennal response was followed in many instances by a more vigorous response in which the lobster turned to face the direction of the water jet, gave a tailflip escape response, or both. Field water jets also produced higher overall response percentages, *i.e.*, 95% versus 88% for laboratory water jets.

Behavioral variability may result from factors such as the size difference of the animals used in the laboratory and field studies. Although larger animals respond with more vigorous turn or escape responses, this is not pre-

dicted by the developmental differences displayed in the clawed lobster *Homarus* where escape responses in small animals give way to defensive displays in larger animals (Lang *et al.*, 1977). However, the ontogeny of defensive behavior in *Homarus* is associated with an allometric increase in claw size. The spiny lobster has no chelae. Alternatively, the absence of escape responses under laboratory conditions may be at least partly a result of restraint-induced inhibition of escape behavior, as demonstrated previously in the crayfish (Krasne and Wine, 1975). Even though restraint in our laboratory experiments allowed simulated walking, animals nonetheless experience less freedom of movement than foraging animals. In addition, turns and tailflips may be due in part to a more suprathreshold level of stimulation associated with hand-delivered stimuli in the field.

Mechanosensory-induced antennal responses, along with turns and tailflips, indicate the ability of spiny lobsters to localize (within 30°) the source of hydrodynamic signals, and to respond selectively to direct contact with their body surface. Water jets, regardless of direction or intensity, trigger bilateral antennal movements. The bilateral hydrodynamic response characteristically involves same-direction movement of both antennae, with the contralateral antennal response statistically indistinguishable from that of the ipsilateral antenna (Fig. 5). In effect, both antennae sweep toward a stimulus directed from the front or rear of the animal. The ipsilateral antenna also moves toward a lateral stimulus but the contralateral response is seemingly anomalous, essentially pointing in the opposite direction. Nevertheless, the bilateral antennal response reflects activation by the hydrodynamic stimulus of a receptive field encompassing both sides of the animal, in comparison to the more localized tactile stimulus, and suggests that there is strong bilateral coupling of direction-specific neural elements underlying the antennal behavior.

On the other hand, threshold-level touch elicits exclusively ipsilateral antennal movements (Fig. 7). The unilateral response to a more localized tactile stimulus indicates that antennal motor circuits also exhibit independent, uncoupled control of the appendage. For responses to light touch, stimulus lateralization is absolute. In foraging, unrestrained animals, however, tactile stimulation again elicits a bilateral response in which both antennae sweep the same direction. As with hydrodynamic stimulation, field stimuli are apparently suprathreshold and trigger tailflips along with antennal responses (Fig. 8).

The direction and degree of antennal sweep responses are nonetheless clearly related to stimulus direction or location and direct the antennae toward the stimulus. Water jets from in front of the animal, broadly defined by a $\pm 60^\circ$ arc, trigger almost exclusively forward sweeps of the antennae (Fig. 5), and these differ significantly

from backward movements for all other stimulus directions (Figs. 5 and 6).

Tactile responses also broadly distinguish between anterior and posterior stimulus sites. Contact with the antennules or the medial edge of the antennal flagellum produces forward rotation; all other sites trigger backward rotation. As with hydrodynamic stimuli light touches trigger a relatively weak response, with a maximum mean amplitude of -14° for carapace stimulation (Fig. 7). Except for direction, stimulus site has little effect; that is, response amplitude does not vary significantly according to the relative anterior-to-posterior location of the stimulus, at least for light contact. However, the distinction between an anterior and posterior tactile stimulus is particularly clear at the level of the antenna, where medial and lateral sites trigger responses of opposite direction. The antennal stimuli used here may activate proprioceptive resistance reflexes (*cf.* Vedel, 1980) in addition to responses mediated by tactile receptors; responses mediated by thoracic and abdominal inputs would originate primarily from tactile and hydrodynamic receptors.

Comparisons of antennal responses in palimurid and astacid decapods

The behavior of the antennal flagellum in the spiny lobster *P. argus* is different in a number of respects from that in the chelate decapod crustaceans. In this study, where lobsters were observed in both active and inactive states, the antennae were held in a lateral resting posture perpendicular to the long body axis. Resting position may vary with circumstance: for example, in small lobsters (<20 mm carapace length in our studies) the flagella are often held vertically, and the animals themselves are less likely to occupy flat, horizontal benthic substrates. In crayfish, as with *P. argus*, the resting antennal position is also near horizontal, but the flagella point in a more rostral to rostrrolateral direction, *e.g.*, 20° – 60° in *Orconectes limosus* (Tautz, 1987), 20° – 50° in *Cherax destructor* (Zeil *et al.*, 1985), and 50° in *Procambarus clarkii* (Schmitz, 1992). The lobster *Homarus americanus* is exceptional in that the antennae at rest are held fully flexed backward alongside the cephalothorax (Sigvardt, 1977). The backward position in *Homarus* correlates with the prominent chelipeds, which not only present a defensive barrier but also contain many mechanosensory receptors (Derby, 1982). In crayfish, the forward resting position presumably emphasizes the mechanosensory function of the antennae, whereas in the spiny lobster a more lateral position may be optimal for combined sensory and defensive functions.

Antennal responses to hydrodynamic stimuli in crayfish are in general similar to the bilateral responses re-

ported here for the spiny lobster, although some differences have been noted. Surface vibrations from a dipole oscillator positioned laterally elicit bilateral antennal movements in *O. limosus* (Tautz, 1987), but the antennae respond independently, the ipsilateral antenna moving toward the stimulus while the contralateral antenna moves in either direction. A dipole oscillation is a more complex stimulus (Wilkens and Douglass, 1994) and may account for the variable response of the contralateral antenna. Schmitz (1992) employed water jets to stimulate primarily the abdominal region in *P. clarkii* and, as in *P. argus*, a bilateral, same-direction response was elicited. However, with jets centered on the tailfan and limited primarily to lateral and caudal sectors (equivalent to those defined in the present study), only backward responses were observed. The antennal movements in *P. clarkii* also demonstrate lateralized sensitivity for particular stimulus directions—rostrrolateral when using stronger water-jet currents, and lateral to caudolateral for weaker stimuli. By reversibly blocking hydrodynamic receptors on the tailfan, Schmitz (1992) also demonstrated the importance of tailfan mechanoreceptors for more cephalic reflex behaviors. Lateralization was clearly evident in *P. argus* for the most caudal stimulus directions but, because our stimuli were centered on the mid-thorax region, the receptive fields affected were somewhat different. As in crayfish, however, in our spiny lobster, the tailfan played a prominent role as a sensory system in that antennal responses were largest and showed significant lateralization when the stimulus was aimed at the tailfan, *i.e.*, in the caudal sector.

Among crayfish and lobsters, responses to tactile stimuli show greater variability than to hydrodynamic stimuli, and the adaptive significance is not always clear. As reported here, the spiny lobster moves one (ipsilateral) or both of its antennae backward for most stimulus sites, the exceptions being the antennules and the medial edges of the antennae. In two Australian crayfish, *Euastacus armatus* (Sandeman and Wilkens, 1983) and *C. destructor* (Sandeman, 1985), however, tactile stimulation of the branchiostegite (carapace) elicits strong, forward extensions of the ipsilateral antenna. Cephalic (tegumentary receptive fields) stimuli sometimes produce backward sweeps in *Euastacus*. Touching or pinching the telson and uropods of these crayfish, as with *P. argus*, triggers flexion of the flagellum in an apparent rotation of the appendage toward the site of potentially threatening contact. The response is similar for *O. limosus* but, as for dipole stimulation, the contralateral antenna moves variably in either direction (Tautz, 1987).

Rostral tactile stimulation also yields different responses. In the spiny lobster, a tactile stimulus "in front" of the animal triggers a forward, reflex extension of the

ipsilateral antenna. In the chelate astacids the response is opposite. In *Cherax* the antenna withdraws (flexes) when touched (Sandeman and Varju, 1988), and in *Homarus* disturbances in front of the animal cause the antennae to assume the "resting," backward-pointing position (Sigvardt, 1977). Touching the rostrum in *Orconectes* has a mixed effect (Tautz, 1987). Backward sweeping of the flagellum in crayfish is apparently associated with, and precedes, aggressive behavior. For example, touching the tip of the antennae in *Cherax* triggers withdrawal of the antennae, after which the crayfish turns, moves forward, and grasps with the chelae (Zeil *et al.*, 1985; Sandeman and Varju, 1988).

Natural history of antennal behavior

The functional anatomy of the spiny lobster antennae suggests strongly that they assume a defensive role not shared by the antennae of chelate decapods. The behavioral responses described in the present study, in which movements in response to both hydrodynamic and tactile stimuli are toward the source of the stimulus signal, support this observation. It is not known if pointing the flagellum toward the stimulus enhances mechanosensitivity, as is postulated for the crayfish (Tautz, 1987), but the benefit of aiming the stiff, spiny flagellum at a potential predator is clear. The mechanosensory response is essentially equivalent to visually mediated movements of the antennae, although the latter have not been quantified, and therefore would be synergistic with or act in lieu of visual guidance in the dark.

The natural history of spiny lobsters suggests several functional interpretations for the observed antennal responses, especially for animals on open substrate at night. The primary nocturnal predators of juvenile lobsters are fish (*e.g.*, snappers, grouper) and sharks (*e.g.*, bonnet head and nurse sharks) (Smith and Herrnkind, 1992). These large, actively swimming predators are likely to be detected first by water pulses (*e.g.*, as measured by Bleckmann *et al.*, 1991), not unlike our water jets, caused by water turbulence as they pass close by or, especially, turn and circle the potential prey. The high response rate (95 percent) to water jets and the nature of the response—frequent tailflipping and rapid turning toward the jet's source (Table I)—indicate an active escape response or preparation for defense. By both directing the antennae and turning the body toward the stimulus, a lobster presents its best-armored body regions (rostral horns and spine-studded antennal bases) and its fencing weapons (antennal flagella) to the potential attacker. Simultaneously, the far more vulnerable abdomen is better protected and readied for propulsion away from the attack. This also may explain the higher responsiveness to the more posterior water jets and tactile stim-

uli; these induced the largest bilateral antennal sweeps and the most tailflipping. Bringing both antennae to the stimulated region seems the best tactic to detect and ward off a predator whose next direction of approach cannot be readily predicted.

Responses to tactile stimulation in the field were less frequent and less pronounced than responses to hydrodynamic stimuli, particularly in the anterior body region. This probably resulted in part from the lightness of the stroke we applied, but otherwise represents the normal response of lobsters we observed being brushed by seagrass blades, sea whips, and other common objects moved by water currents as lobsters forage and move about. In contrast, a large fish is less likely to brush against only a small body area. However, a strong hydrodynamic stimulus almost certainly signifies a large swimming animal, many of which are potential predators, as discussed above.

In this study we did not examine other potential antennal functions or responses. The antennae are not known to be involved in feeding; antennaless individuals forage normally using the antennules and pereopods (pers. obs., W.F. Herrnkind). They may have a limited role in social communication (Lipcius *et al.*, 1983), and their position is regulated during locomotion depending on walking speed (Bill and Herrnkind, 1976). That is, while slowly walking and foraging, they are held more-or-less perpendicular to the body or slightly forward. As speed increases, both antennae are swept further forward, thus decreasing hydrodynamic drag, as often seen in mass queuing of migrating lobsters (Bill and Herrnkind, 1976). How other stimuli influence antennal responses in such situations is not yet known. The high level of responsiveness and clear patterns from our field study of free-moving lobsters invites further research. In particular, measurements of hydrodynamic features of moving lobster predators modeled by more controllable devices might give insight into both the physiological processes and the events to which they are tuned evolutionarily.

By contrast, in crayfish and *Homarus* the antennae are involved in the behaviors of communication, *e.g.*, antennal whipping among conspecifics (Rubenstein and Hazlett, 1974; Solon and Cobb, 1980), exploratory activities (Sandeman and Varju, 1988; Zeil *et al.*, 1985), and agonistic and feeding behaviors (reviewed by Voigt and Atema, 1992). The active role played by the crayfish antennae in prey localization has been shown recently using natural stimuli from live fish (Breithaupt *et al.*, 1995). In these experiments, reversibly blinded crayfish (*P. clarkii*) react to hydrodynamic disturbances produced by small swimming fish with accurate body turns and cheliped movements that result occasionally in prey capture. The orientation responses are always preceded

by antennal sweeps, with both antennae moving toward the stimulus. In contrast to the spiny lobsters analyzed here using artificial stimuli, the ipsilateral antennal movements of the crayfish are usually directed backward whereas those of the contralateral antenna are smaller and directed forward, often resulting in antennal contact with the fish. We do not know yet whether spiny lobsters facing a live fish (be it predator or prey) respond in a similar way. In these nonchelate decapods, however, moving the well-armed antenna toward the stimulus source would be an excellent way to gain more information about the location and size and nature of a potential predator, as well as to present a defensive front.

Acknowledgments

This project was supported by a Research Award from the University of Missouri—St. Louis. We also enjoyed the use of laboratory facilities in the Department of Biological Science and Marine Laboratory, Florida State University, and at the Keys Marine Laboratory, Layton, Florida, of the Florida Institute of Oceanography. We thank Dr. Xing Pei and Dr. David Russell for assisting with computer graphics. A publication of the Tallahassee, Sopchoppy and Gulf Coast Marine Biological Association (No. 314).

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Capture of Large Particles by Suspension-Feeding Scaleworm Larvae (Polychaeta: Polynoidae)

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Abstract. Most of the polychaete larvae in which feeding mechanisms have been studied feed using an opposed-band mechanism, capturing particles with prototrochal and metatrochal ciliary bands and transporting them to the mouth *via* a food groove. However, many other planktotrophic polychaete larvae lack a metatroch and food groove and thus must feed in a different way. In this latter group are the larvae of polynoid polychaetes, which not only lack a metatroch and food groove but also bear a bundle of long cilia (the oral brush) attached near the left side of the mouth. In feeding experiments with polystyrene beads and plankton, larvae of the polynoid *Arctonoe vittata* ingested larger particles (up to 60 μm in diameter) than those ingested by the opposed-band feeding larvae of the serpulid *Serpula vermicularis* (up to 12 μm in diameter). Videotaped images of feeding *A. vittata* larvae showed that capture behavior was elicited as particles in a feeding current driven by the prototroch approached or contacted the larval episphere. Particles on or very near the episphere were disengaged by a recoiling motion of the larva and were then moved to the mouth, probably by the oral brush. This feeding mechanism may be widespread in the polychaete superfamily Aphroditacea, which includes about 10% of extant polychaete species.

Introduction

The feeding mechanisms of polychaete trochophore larvae that have three equatorial ciliary bands (the prototroch, food groove, and metatroch) are relatively well understood (R.R. Strathmann, 1987). The long cilia of the prototroch beat from anterior to posterior, producing

a current for both swimming and feeding. Some of the particles that pass within reach of the prototrochal cilia are swept into the food groove, possibly with assistance from the metatrochal cilia (which beat from posterior to anterior). Particles retained in the food groove are carried to the mouth. This "opposed-band" mode of particle capture has been documented in larvae of serpulid and oweniid polychaetes as well as in larvae of bivalve and gastropod molluscs (Strathmann *et al.*, 1972; Strathmann and Leise, 1979; Lacalli, 1984; Gallagher, 1988; Emlet and Strathmann, 1994). Work on the larvae of capitellid polychaetes and bivalve molluscs (Riisgård *et al.*, 1980; Fritz *et al.*, 1984; Sprung, 1984; Hansen, 1993) suggests that opposed-band feeders capture small particles ($<10\ \mu\text{m}$) far more efficiently than large ones. This upper limit on the sizes of captured particles may be imposed by the dimensions of the feeding structures, in particular the length of the prototrochal cilia and the width of the food groove (R.R. Strathmann, 1987).

Many planktotrophic polychaete larvae, however, lack a metatroch and a food groove and thus must capture and handle particles differently than do opposed-band feeders. Such larvae (*e.g.*, glycerids, nephtyids, and phyllodocids) are often among the most abundant polychaete larvae in temperate waters (Lacalli, 1981; Yokouchi, 1991), but their feeding behavior is unknown. Larvae of polychaetes in the superfamily Aphroditacea (scaleworms) not only lack a metatroch and food groove, but also bear a single bundle of long cilia attached near the prototroch at the left side of the mouth and extending posteriorly (Fig. 1a, b, c). We will refer to this asymmetrically placed bundle of cilia as the oral brush, after Lacalli (1980). Whether the oral brush is involved in feeding is not known. Also unknown is how the feeding mechanism of scaleworm larvae might influence the size range of particles that they can capture and handle. The

Received 9 February 1996; accepted 8 July 1996.

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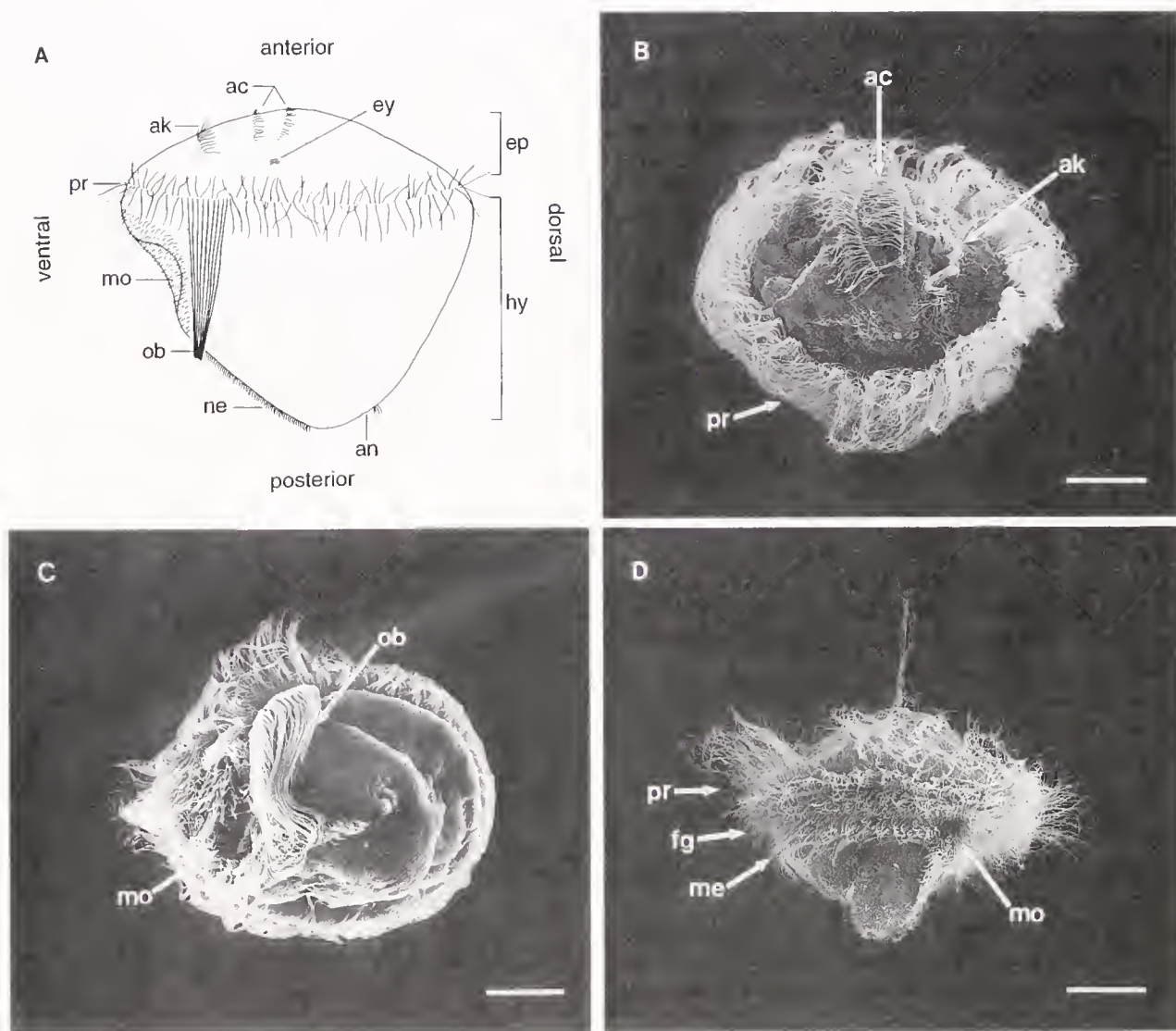


Figure 1. Larvae of *Arctonoe vittata* and *Serpula vermicularis*. (A) Line drawing of *A. vittata* larva, in left lateral view. (B) Scanning electron micrograph of 27-day-old *A. vittata* larva, in anterior view; the ventral side of the larva is to the right. Scalebar = 25 μ m. (C) Scanning electron micrograph of 26-day-old *A. vittata* larva, in posterior view; the ventral side of the larva is to the left. Scalebar = 25 μ m. (D) Scanning electron micrograph of 8-day-old *S. vermicularis* larva, in right lateral view. Scalebar = 25 μ m. ac = apical cilia, ak = akrotoch, an = anus, ep = episphere, ey = eye, fg = food groove, hy = hyposphere, me = metatroch, mo = mouth, ne = neurotoch, ob = oral brush, pr = prototroch.

guts of scaleworm larvae captured from the plankton often contain particles much larger than 10 μ m in diameter (e.g., diatoms and bivalve veligers 30–100 μ m in diameter: Lebour, 1922; Lacalli, 1981; Yokouchi, 1991).

In this paper we describe feeding in larvae of the polynoid scaleworm *Arctonoe vittata*. We compared the sizes of artificial and natural particles ingested in the laboratory by larvae of *A. vittata* and by similarly sized larvae of the serpulid *Serpula vermicularis*. Larvae of *S. vermicularis* have a prototroch, metatroch, and food groove, and they feed with the opposed-band mechanism de-

scribed above (Strathmann *et al.*, 1972; Fig. 1d). In our experiments, *S. vermicularis* larvae fed only on particles 12 μ m or less in diameter, a result consistent with the generalization that opposed-band feeders capture small particles more efficiently than large ones. *A. vittata* larvae, on the other hand, ingested particles up to 60 μ m in diameter. Analysis of videotaped sequences of feeding *A. vittata* larvae showed that they used the prototroch to generate a feeding and locomotory current and the ciliated episphere to sense and capture particles. Captured particles were then transferred to the mouth, probably

by the oral brush. This previously undescribed feeding mechanism is probably widespread within the aphroditaceans, most of which have planktotrophic larvae with oral brushes.

Materials and Methods

Larval cultures

Adult specimens of *Arctonoe vittata* were collected with host keyhole limpets (*Diodora aspera*) in the intertidal zone on the west side of San Juan Island, Washington. Specimens of *Serpula vermicularis* were collected from Argyle Creek, San Juan Island. Spawning was induced in females of both species on 18 June 1995 by removing individuals from their hosts or tubes, respectively, and isolating them in small dishes of seawater. Oocytes spawned by one female of each species were briefly rinsed in 0.45 μm filtered seawater (FSW) and inseminated with sperm removed from the coelomic cavity of a single conspecific male. More than 90% of the eggs in each cross were fertilized. Larvae were cultured in 10 μm FSW at densities of approximately 200 larvae $\cdot\text{l}^{-1}$. The cultures were stirred with a system of swinging paddles (M. Strathmann, 1987), and the culture jars were kept partially immersed in a seatable at 10°–13°C (near local ambient sea temperature). All larvae used in feeding experiments were starved for 12 h prior to the experiment; otherwise they were fed a mixture of *Rhodomonas* sp. (maximum dimension about 10 μm) and *Isochrysis galbana* (maximum dimension about 6 μm) every 3–5 days, immediately after water changes.

Sizes of ingested particles: polystyrene beads

This experiment was conducted to determine the size range of particles that larvae of each species would ingest. In preliminary experiments, larvae were fed suspensions of single size classes of beads (2, 10, 20, and 40 μm) and a single mixed suspension of these size classes Phillips (unpub. data). The size ranges of beads ingested by larvae in both of these experimental conditions were the same. Therefore, for the feeding experiment described here we used a single mixed suspension of multiple size classes of beads. We used spherical beads of diameters we shall refer to as 2, 10, 20, and 40 μm (the manufacturer's stated diameters were 2.12 μm [Duke Scientific, Bioclean, green-fluorescing], 9.87 $\pm$ 0.57 μm , 19.5 $\pm$ 0.6 μm , and 42.1 $\pm$ 0.8 μm [Duke Scientific, Size Standards, NBS Traceable]). We made stock suspensions of each bead size class in 0.45 μm FSW and made 6–10 replicate counts per size class in a hemacytometer (2-, 10-, 20- μm beads) or a Bogaroff tray (40- μm beads) to estimate concentrations. To eliminate clumps of beads, each suspension was placed in a bath sonicator for 5 min prior to

counting. From these stocks we made a working mixed suspension consisting of 8340 2- μm beads $\cdot\text{ml}^{-1}$, 4170 10- μm beads $\cdot\text{ml}^{-1}$, 2085 20- μm beads $\cdot\text{ml}^{-1}$, and 417 40- μm beads $\cdot\text{ml}^{-1}$.

The bead-feeding experiment was conducted 8 days after fertilization. Larvae of *A. vittata* were distributed among five 20-ml glass vials (20 larvae/vial), and larvae of *S. vermicularis* were distributed similarly among another five vials. Aliquots (15 ml) of sonicated working suspension were introduced into each vial. To maintain particles in suspension, these vials were strapped to a revolving plankton wheel (0.025 revolutions $\cdot\text{s}^{-1}$, diameter = 23 cm) and maintained at 12°C. Preliminary experiments suggested that larvae of the two species ingested particles at different rates (Phillips, unpub. data); therefore, *S. vermicularis* larvae were removed from the plankton wheel after 30 min, and *A. vittata* larvae were removed after 1 h. Larvae were killed immediately by the addition of formalin to a final concentration of approximately 5%.

The gut contents of as many larvae as could be recovered from each vial (15–19) were examined using an epifluorescence microscope. A fluorescein isothiocyanate filter (ex. = 420–490 nm, em. = 520 nm) was used to detect the 2- μm beads. All other beads were detected with normal brightfield optics. Beads were counted as ingested if they were found anywhere in the digestive system of the larva, and the number and sizes of beads ingested by each larva were recorded.

Sizes of ingested particles: concentrated natural plankton

To corroborate the results of the bead-feeding experiment, we conducted additional feeding experiments using natural plankton. We obtained concentrated natural plankton by towing a 25- μm -mesh net vertically off the breakwater at the Friday Harbor Laboratories. The concentrated plankton was passed through a 200- μm mesh to remove large particles and predators. Eight-day-old *A. vittata* larvae were placed into two 20-ml vials (20 larvae/vial), and 8-day-old *S. vermicularis* larvae were similarly divided between another two vials. Fifteen milliliters of concentrated plankton was added to each vial, and the vials were strapped to the plankton wheel used in the bead experiment, at 12°C. After 3 h larvae were examined alive (so that ingested particles could be better identified), immobilized in a suspension of polyethylene oxide in seawater, and the largest particles each had ingested were measured. Ten larvae of *A. vittata* from each of the two vials and 10 larvae of *S. vermicularis* from a single vial were examined. A second plankton-feeding experiment was conducted with 18-day-old larvae (15 of each species) under identical conditions.

Feeding by larvae of *Arctonoe vittata*: videotaped observations

Larvae of *A. vittata* maintained in unstirred cultures tend to become tethered to the bottom of the culture vessel by strands of mucus trailing from their posterior ends. We videotaped such tethered larvae in suspensions of particles to visualize particle capture and handling. About twenty 27- to 30-day-old *A. vittata* larvae from unstirred cultures were gently transferred to a small petri dish filled with 0.45- μm FSW. A suspension of 40- μm polystyrene beads was added, and the dish was covered with a glass microscope slide, excluding air pockets. Larvae in the dish were examined with a dissecting microscope equipped with a Sony HVM-200 video camera linked to a video recorder and monitor. Larvae that were tethered (little or no forward motion) but that continued to rotate normally were videotaped at a magnification of 40 $\times$. Larvae and seawater were changed every 30 min to minimize the effects of temperature increases. Although tethering undoubtedly influenced patterns of flow around larvae (e.g., Emlet, 1990), it probably did not affect qualitative aspects of the mode of feeding that we describe.

Measurements of larvae

Eight-day-old and 18-day-old larvae were immobilized with a small amount of polyethylene oxide in seawater and examined alive with a compound microscope.

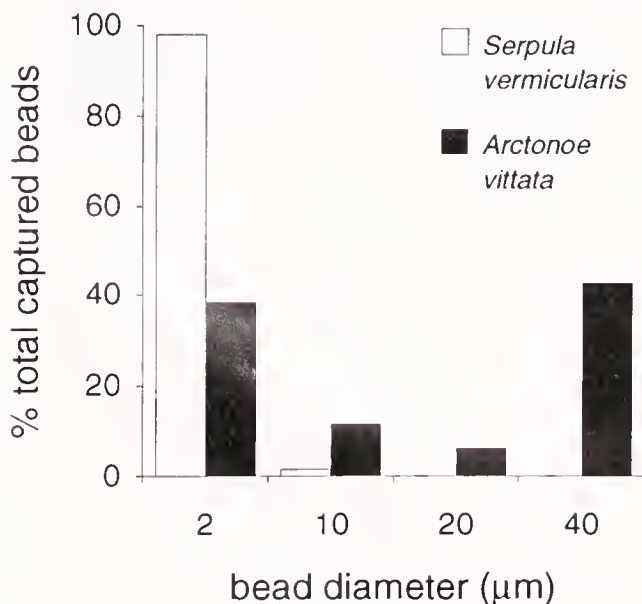


Figure 2. Percentage of beads in each size class captured by 8-day-old larvae of *Arctonoe vittata* and *Serpula vermicularis* in the bead-feeding experiment. Fifty-four *A. vittata* larvae ingested a total of 119 beads; fifty-two *S. vermicularis* larvae ingested a total of 116 beads.

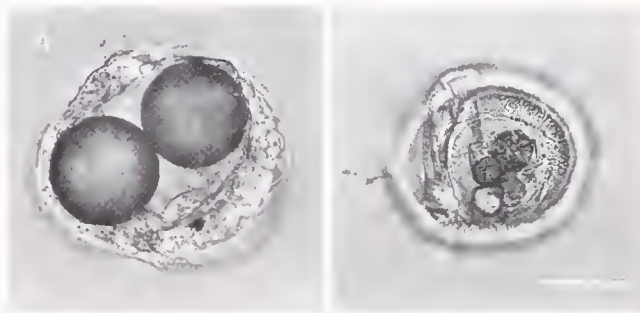


Figure 3. Light micrographs of *Arctonoe vittata* larvae that ingested particles in feeding experiments. Figures A and B are not to the same scale. (A) An 8-day-old larva (killed in formalin) with two polystyrene beads in its gut. Each bead is 40 μm in diameter. (B) An 18-day-old larva (live) in posterior view with several large particles from natural, concentrated plankton in its gut. Scalebar = 50 μm .

For larvae of both species we measured total body length, maximum body width (equivalent to prototroch diameter), prototrochal cilium length, and mouth width. In addition, we measured the width of the food groove in larvae of *S. vermicularis* and the length of the oral brush in larvae of *A. vittata*. Because it was difficult to manipulate larvae in polyethylene oxide, not all measurements were made on each individual. We used a one-way analysis of variance and *posthoc* tests (Scheffé's *F*-test; Sokal and Rohlf, 1981) to identify significant ($P < 0.05$) differences in size among the four groups of larvae measured.

Results

Sizes of ingested particles: polystyrene beads

Seventy-six larvae of *A. vittata* were recovered and examined. Of these, 54 (71%) had ingested 1–6 beads, and the remainder had not ingested any. Of 82 larvae of *S. vermicularis* 51 (62%) had ingested 1–12 beads, and the remainder had not ingested any. *A. vittata* larvae ingested beads over the whole size range offered, though most beads ingested were in the 2- and 40- μm size classes (Figs. 2, 3a). *S. vermicularis* larvae ingested mostly 2- μm beads, and they ingested no beads greater than 10 μm in diameter (Fig. 2).

Sizes of ingested particles: concentrated natural plankton

In the first plankton-feeding experiment, with 8-day-old larvae, all 20 *A. vittata* larvae and all 10 *S. vermicularis* larvae examined had ingested 1–10 particles ranging in maximum diameter from 3–12 μm ; no larvae of either species had any larger particles in their guts.

In the second plankton-feeding experiment, with 18-day-old larvae, 15 larvae of each species were examined. Twelve of the 15 *A. vittata* larvae (80%) had ingested

from 1 to 5 particles 30–40 μm in diameter. These were mostly dinoflagellates (Fig. 3b). One *A. vittata* larva had ingested a 90- μm -long pennate diatom as well as a centric diatom 60 μm in diameter. Most of these larvae had also ingested many other unidentified particles smaller than 10 μm in diameter. Ten of the 15 *S. vermicularis* larvae (66%) had ingested visible particles; these were all smaller than 6 μm in maximum diameter.

Feeding by larvae of Arctonoe vittata: videotaped observations

We videotaped 10 particle captures by five different larvae. In five instances the larvae captured and ingested 40- μm beads; in three instances the larvae captured and rejected 40- μm beads; and in the remaining two instances larvae responded to small (<10 μm) particles present as contaminants. In these last two cases it was not possible to determine if the particles were successfully captured and ingested. The events of particle capture were similar in all instances observed.

The sequence of events in the capture and ingestion of a 40- μm bead is shown in Figures 4 and 5. The tethered larva rotated clockwise (viewed anteriorly) around its anterior-posterior axis at about 1 revolution $\cdot\text{s}^{-1}$, the prototroch creating a current in the anterior to posterior direction. A particle entrained in this current rapidly approached the larva (Figs. 4a, 5a), and came very near to or contacted the central portion of the episphere (Figs. 4b, 5b). The particle then moved ventrally on the episphere, towards the mouth of the larva (Figs. 4c, 5c). At this point the larva recoiled briefly, moving its entire body such that the particle was disengaged from the episphere (Figs. 4d, 5d). After the recoil, the larva continued its clockwise rotations. The bead, now located 80–90 μm ($n = 3$ captures) ventrally and slightly to the left of the mouth, rotated with the larva as if it was being held in that position (Figs. 4e–g, 5e–g). The larva and bead rotated together for 0.3–2 revolutions before the bead was seen at the mouth (Figs. 4h, 5h) and soon thereafter the bead was visible in the gut of the larva (Figs. 4i, 5i). The larva continued to rotate normally while the captured bead was held in front of the mouth and then ingested. We were unable to see the oral brush during particle capture or handling.

In all three instances in which a 40- μm bead was captured and then rejected, the events of capture were as described above. When rejection occurred it was always after the bead had been held in the mouth (as in Fig. 4h) for one or several revolutions. In two cases, the rejected bead slowly moved down the neurotroch before being released near the anus. In the other case the rejection path of the bead was not visible because of the orientation of the larva.

Measurements of larvae

Larvae of *A. vittata* increased in mean length, body width, prototrochal cilium length, and oral brush length between 8 and 18 days after fertilization (Table 1). Larvae of *S. vermicularis* decreased in body width over this time period. Mouth width did not change significantly in larvae of either species between 8 and 18 days after fertilization. Eight days after fertilization, larvae of *A. vittata* were smaller in length and diameter than larvae of *S. vermicularis*; these differences were no longer apparent 18 days after fertilization. Mouth width and prototrochal cilium length in *A. vittata* larvae were significantly greater than in *S. vermicularis* larvae at both 8 and 18 days after fertilization.

Discussion

The results of the bead- and plankton-feeding experiments demonstrate that the polynoid and serpulid larvae we observed ingest particles of different size ranges. The opposed-band feeding larvae of *Serpula vermicularis* ingested only small particles ($\leq 12 \mu\text{m}$) in all experiments, whereas larvae of *Arctonoe vittata* ingested particles 2–60 μm in diameter. Only in the first plankton-feeding experiment, with 8-day-old larvae, did larvae of the two species ingest particles of similar sizes (all $\leq 12 \mu\text{m}$). That result may reflect the absence of larger particles in the plankton on that day. This interpretation is supported by the results of a bead experiment carried out simultaneously; here many 8-day-old *A. vittata* larvae ingested 40- μm polystyrene beads (Fig. 2). We did not determine the size distribution of particles in the plankton and cannot address this problem directly. For larvae of *S. vermicularis*, our results are consistent with the hypothesis that the dimensions of the prototrochal cilia or food groove set an upper limit on the sizes of particles that can be captured or handled (R.R. Strathmann, 1987). The mean sizes of both of these structures were larger than the maximum size of ingested particles in all feeding experiments.

Direct observations confirmed that the events of feeding by larvae of *A. vittata* are unlike those described for other trochophores. In tethered larvae of *A. vittata*, some of the particles carried in the feeding current approached very close to the surface of the episphere, and others appeared to directly strike the episphere (Figs. 4b, 5b). In all of the captures we observed, particles approached or contacted the central or ventral region of the episphere. Either the close approach or contact of particles with the episphere was sufficient to stimulate capturing behaviors. At this stage the particle may have simply been sensed by the larva, or it may have actually been captured on the surface of the episphere. The epispheres of many polynoid larvae, including those of *A. vittata*, bear

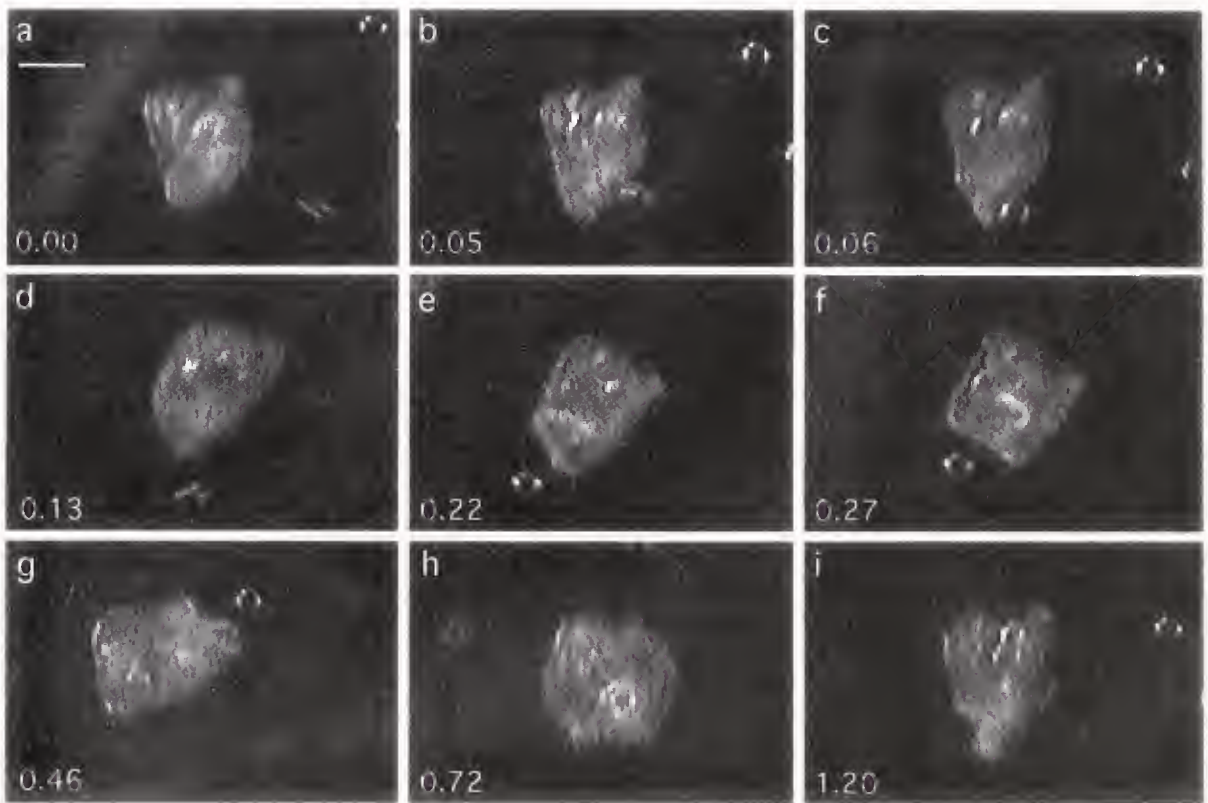


Figure 4. A sequence of videotape frames showing the capture of a 40- μ m bead by a swimming, self-tethered *Arctonoe vittata* larva. Time (seconds) is in the bottom left corner. All images are at the same magnification; the scalebar is 100 μ m. The larva is rotating around its anterior-posterior axis in a clockwise direction (when viewed from the anterior), and swimming towards the lower right of the image. (a) A bead approaches the larva's episphere. (b) The bead contacts the episphere. (c) The bead moves toward the upper lip. (d) The larva recoils and the bead is disengaged from the episphere. (e–g) The larva begins to rotate again, carrying the bead in front of and slightly to the left of the mouth. (h) After one full revolution the bead is held in the mouth. (i) The bead (as well as a previously captured bead) is visible in the gut.

several transverse ciliary bands (Fig. 1a, b; Cazaux, 1968; Holborow, 1969; Lacalli, 1981). In larvae of *A. vittata* (and larvae of *Harmothoe imbricata*; Holborow, 1969), the two bands located nearest to the episphere apex beat slowly and discontinuously; the band of cilia located between the episphere apex and the mouth (the "akro-troch," Fig. 1a, b) beats rapidly and continuously in the direction of the mouth (Pernet, pers. obs.). These ciliary bands may act in feeding by sensing approaching particles, distributing adhesive secretions over the episphere, or moving particles ventrally towards the mouth of the larva.

After the particle had been moved ventrally on the episphere, the tethered larva recoiled rapidly and then continued rotating. This recoiling motion, presumably a consequence of a brief reversal of beat of the prototrochal cilia, disengaged the particle from the region of the episphere, leaving it positioned about 80–90 μ m ventral to and slightly to the left of the mouth (Figs. 4d–e, 5d–e).

As the larva continued rotating, the particle remained in this position relative to the larval mouth, as if it was being held there (Figs. 4e–g, 5e–g). Based on these observations, we suggest that after the particle is disengaged from the episphere, the oral brush (normally carried parallel to the anterior-posterior axis of the body) is flung out ventrally where the particle is recaptured on its tip. Though lighting and limited magnification did not permit a clear view of the oral brush during captures, no other larval structure appears capable of effecting the observed movements of captured beads. The oral brush is attached on the left side of the mouth and is long enough to contact particles 80–90 μ m distant (Table I; in the 27- to 30-day-old larvae used in these observations the oral brush was probably longer than 90 μ m). How the particle might be held by the oral brush, or moved to the mouth, is not known. Perhaps the oral brush adheres to the particle, or perhaps the particle is held there by flow around it.

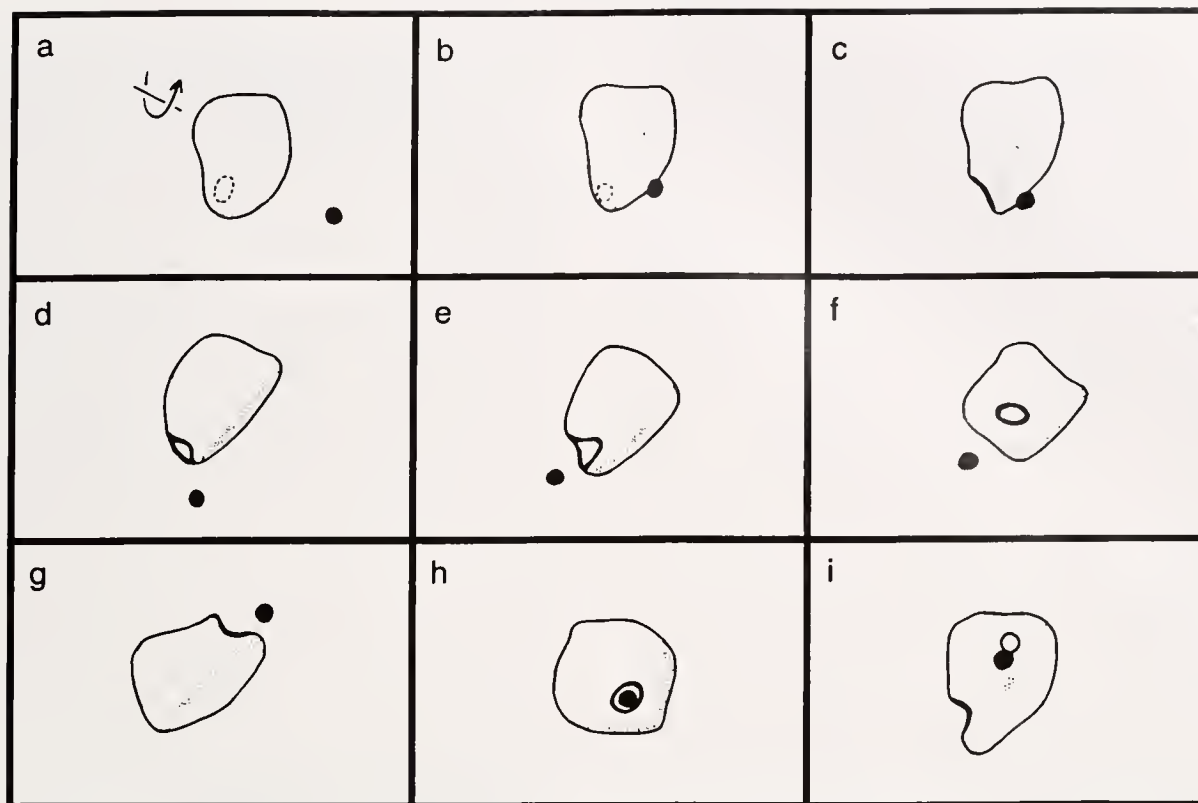


Figure 5. Line drawings of the video sequence shown in Figure 4; see the legend for that figure for details. The axis and direction of rotation of the larva is shown in (a). The stippled line represents the prototroch; the black sphere is the bead being captured; a previously ingested bead is represented as an open circle in (i). In (a) and (b) the mouth, on the opposite side of the larva from the observer, is represented by a dashed line. As the mouth comes into view (c–i) it is outlined with a heavy line. The oral brush is not shown.

The feeding mechanism we describe resulted in the capture of 40- μm particles. However, larvae of *A. vittata* also captured many smaller particles in feeding experiments (e.g., Fig. 2). It is unlikely that these were ingested in the form of larger clumps of associated particles, because many larvae had ingested only one or a few small

particles. Also, larvae in cultures captured and ingested the relatively small ($\leq 10\ \mu\text{m}$) algal cells that they were fed. Videotape observations showed that larvae responded to these particles with behaviors similar to those exhibited in response to 40- μm particles. However, because these small particles were difficult to see, we were

Table I

Sizes of Serpula vermicularis and Arctonoe vittata larvae at 8 and 18 days post-fertilization

	<i>Serpula vermicularis</i>		<i>Arctonoe vittata</i>	
	8 days old	18 days old	8 days old	18 days old
Body length	115.2 $\pm$ 9.3 (15)	123.7 $\pm$ 10.2 (7)	94.2 $\pm$ 6.1 (13)	129.1 $\pm$ 12.6 (8)
Body width	119.7 $\pm$ 7.9 (11)	93.6 $\pm$ 10.5 (7)	89.6 $\pm$ 11.8 (13)	112.4 $\pm$ 8.7 (7)
Mouth width	19.2 $\pm$ 2.9 (13)	16.2 $\pm$ 3.6 (5)	32.4 $\pm$ 4.5 (10)	33.6 $\pm$ 7.1 (3)
Prototrochal cilium length	41.0 $\pm$ 3.8 (15)	40.6 $\pm$ 3.4 (7)	39.5 $\pm$ 4 (11)	51.8 $\pm$ 2.2 (5)
Food groove width	25.3 $\pm$ 2.7 (10)	25.9 $\pm$ 3.9 (7)	—	—
Oral brush length	—	—	63.1 $\pm$ 3.5 (11)	91.7 $\pm$ 2.3 (6)

Mean sizes are reported in μm $\pm$ standard deviations; in parentheses are the number of individuals measured in each category.

unable to discern if they were actually captured and ingested. It is possible that small particles are captured by the same mechanism as large particles. Alternatively, small particles may be captured by the prototrochal cilia in the same way as in opposed-band feeders. In this scenario, only particles captured by prototrochal cilia overlying the densely ciliated oral region (Fig. 1a) might be ingested; the lack of a food groove means that particles captured elsewhere cannot be transported to the mouth. The observation that intermediate-sized beads (10 and 20 μm) were captured less frequently than 2- and 40- μm beads (Fig. 2) supports the hypothesis that *A. vittata* larvae feed using two different mechanisms.

Larvae of *A. vittata* increased in size between 8 and 18 days of age (Table I), and some of these size changes probably had consequences for particle encounter and capture rates. For example, mean body diameter increased from 89.6 to 112.4 μm . This change is reflected by an increase in the cross-sectional area of the episphere. Increases in episphere cross-sectional area imply increases in the volume of water that can be surveyed for particles. The length of the oral brush also increased dramatically between 8 and 18 days of age. A longer oral brush may allow larger particles to be captured, or it may enhance the efficiency of particle handling. Finally, the length of the prototrochal cilia increased between 8 and 18 days of age, from 39.5 to 51.8 μm . In opposed-band feeders, increases in the length of prototrochal cilia are associated with greater angular velocities of cilia, faster movement of water, or higher clearance rates of particles (Emlet and Strathmann, 1994). In larvae of *A. vittata* such increases may result in an increase in the volume of water that is passed by the episphere and surveyed for particles. Though we only measured larvae until 18 days of age, in laboratory cultures larvae of *A. vittata* survive for up to 7–9 weeks, reaching a body diameter of about 300 μm and an oral brush length of about 120 μm (Pernet, pers. obs.). Hence, body-size-associated changes in feeding capabilities may be quite dramatic over the course of development in larvae of *A. vittata*.

There are few obvious constraints on the maximum sizes of particles that can be captured and handled by *A. vittata* larvae. The flexural stiffness of the oral brush may limit the size or density of particles that can be held in front of the mouth. Although the dimensions of the mouth and gut ultimately constrain the sizes of particles that can be ingested, these structures are quite distensible. The mean width of the mouth in *A. vittata* larvae was approximately 33 μm , and larvae ingested polystyrene beads and dinoflagellates up to 40 μm in diameter, and a centric diatom 60 μm in diameter. Neither are there obvious constraints on the minimum sizes of particles that can be captured and handled. It is possible that very small particles may not stimulate episphere sensory

structures and thus may not lead to capture responses (a similar hypothesis was suggested by R.R. Strathmann [1987] to account for the relatively low clearance rates of small particles by echinoderm larvae). The oral brush is composed of very closely spaced cilia (Fig. 1c), and it seems unlikely that even very small particles are able to pass through it.

Although larvae of *A. vittata* can capture and ingest particles of a wide range of sizes, feeding efficiency may vary with particle size. In our bead-feeding experiment, for example, larvae of *A. vittata* ingested 2- and 40- μm beads far more frequently than 10- and 20- μm beads (Fig. 2), despite the fact that they were more likely to encounter the latter sizes in the bead suspension we used. (In direct interception, the mode of particle capture presumably used by these larvae, encounter rate is a function of both particle size and particle concentration [Shimeta and Jumars, 1991]. In our experiment the concentrations of 10- and 20- μm beads were such that they should have been encountered 2.5 times as frequently as 2- and 40- μm beads.) Such patterns may represent constraints imposed by the mechanisms of particle capture and handling. This may help explain the observation of Lacalli (1981) that the abundance of polynoid larvae in Passamaquoddy Bay, New Brunswick, is correlated with the abundance of large diatoms. He suggested that polynoid larvae feed more efficiently on large particles, and that larvae developing earlier or later than the local spring diatom bloom are doomed to high mortality because of a lack of suitably large particles to eat. Other workers have not found a clear correlation between the availability of large particles and the abundance of polynoid larvae (Cazaux, 1973; Yokouchi, 1991). Such linkages between the physical mechanisms of particle capture and the availability of appropriately sized food particles merit further study, and may be useful in understanding some of the selective forces leading to seasonal reproductive strategies in polynoids.

Oral brushes appear to be limited in distribution to larvae of aphroditaceans. The superfamily Aphroditacea includes at least 890 described species (Pettibone, 1982). At least 600 of these species are members of the family Polynoidae, and all known polynoid larvae are planktonic and bear an oral brush (Table II). The only other aphroditacean larvae that have been described belong to the family Sigalionidae, and most of these also lack a metatroch and food groove and bear an oral brush (Table II). Early trochophores of the nephtyid *Nephtys hombergi* have also been reported to bear an oral brush (Wilson, 1936), but other descriptions of this and other nephtyid larvae do not mention the presence of this distinctive bundle of cilia (Rasmussen, 1973; Lacalli, 1980). Larvae that lack a metatroch and food groove and bear an oral brush probably feed as do larvae of *A. vittata*;

Table II

Larval nutritional mode and presence or absence of oral brush in aphroditacean larvae

Family (approximate # of species)	Species	Larval nutrition*	Oral brush	Reference
Aphroditidae (70)	—	—	—	—
Eulepethidae (25)	—	—	—	—
Polynoidae (600)	<i>Acholoe astericola</i>	pl	yes	Davenport, 1954
	<i>Arctonoe fragilis</i>	pl	yes	Pernet, unpub. data
	<i>Arctonoe pulchra</i>	pl	yes	Pernet, unpub. data
	<i>Arctonoe vittata</i>	pl	yes	this study
	<i>Halosydna brevisetosa</i>	pl	yes	Blake, 1975
	<i>Halosydna gelatinosa</i>	pl	yes	Bhaud and Cazaux, 1987
	<i>Halosydna johnsoni</i>	pl	yes	Rossi, 1976
	<i>Harmothoe imbricata</i>	pl	yes	Cazaux, 1968
	<i>Harmothoe impar</i>	pl	yes	Korn, 1958
	<i>Harmothoe longisetis</i>	pl	yes	Cazaux, 1968
	<i>Harmothoe lunulata</i>	pl	yes	Cazaux, 1968
	<i>Harmothoe sarsi</i>	pl	yes	Korn, 1958
	<i>Lagisca extenuata</i>	pl	yes	Cazaux, 1968
	<i>Lepidonotus clava</i>	pl	yes	Cazaux, 1968
	<i>Lepidonotus squamatus</i>	pl	yes	Cazaux, 1968
	<i>Lepidonotus sublevis</i>	pl	yes	Simon, 1965
Pholoididae (15)	—	—	—	—
Polyodontidae (45)	—	—	—	—
Sigalionidae (160)	<i>Pholoe balthica</i>	pl	—	Petersen, cited in Wilson, 1991
	<i>Pholoe inornata</i>	pl	—	Rasmussen, 1956
	<i>Pholoe minuta</i>	pl	no	Heffernan and Keegan, 1988,
	<i>Pholoe minuta</i>	pl	yes	Lacalli, 1980
	<i>Pholoe swedmarki</i>	lec	—	Laubier, 1975
	<i>Pholoe synophthalmica</i>	lec	yes	Cazaux, 1968
	<i>Sthenelais boa</i>	pl	yes	Cazaux, 1968
	"Genus A"	lec	—	Wolf, 1984

* "pl" = planktotrophy, "lec" = lecithotrophy, and "—" = no data. Family-level classification as in Fauchald (1977). No larvae of aphroditids, eulepethids, pholoidids, or polyodontids have been described.

thus the larvae of up to 10% of the 8000 extant polychaete species (Pettibone, 1982) may feed using the mechanism we have described for *A. vittata*.

Because polychaetes use a greater diversity of larval feeding mechanisms than any other major group of marine invertebrates (Strathmann, 1978), and because of the important role that the trochophore larva has played in many phylogenetic schemes (e.g., Nielsen, 1987), the history of evolutionary changes in the feeding mechanisms of larval polychaetes is of particular interest. Developing an understanding of how these mechanisms (e.g., opposed-band feeding and the mode of feeding we describe here for polynoids) are evolutionarily related will require additional data on mechanisms of particle capture in other polychaete larvae, as well as robust phylogenies of the group. A recent phylogenetic analysis of polychaete families places the Aphroditacea within a large group of families whose feeding larvae lack metatroch, food groove, and oral brush (e.g., Glyceridae, Nephtyidae, Phyllodocidae; G. Rouse and K. Fauchald, pers. comm.). These larvae are often found with large

particles in their guts (Lebour, 1922; Mileikovskii, 1959; Lacalli, 1981; Yokouchi, 1991), but how they feed is unknown. Descriptions of particle capture by trochophores that feed without opposed bands and without oral brushes are likely to be particularly useful in assessing patterns of evolutionary change in the feeding mechanisms of larval polychaetes.

Acknowledgments

Parts of this project were conceived in a course in Larval Ecology taught by W.B. Jaeckle and R.R. Strathmann; we thank them for much advice and assistance. The manuscript benefited from criticisms by E. Balser, M. Hart, W.B. Jaeckle, A.J. Kohn, R.R. Strathmann, K. Wasson, A.O.D. Willows, and members of a manuscript discussion group at the Friday Harbor Laboratories, as well as from comments by two anonymous reviewers. We thank the staff of the Friday Harbor Laboratories, University of Washington, for providing laboratory space and equipment. This study was supported by a Na-

tional Science Foundation pre-doctoral fellowship and a grant from the Lerner-Gray Fund for Marine Research to BP.

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Podial Particle Picking in *Cassidulus caribaeorum* (Echinodermata: Echinoidea) and the Phylogeny of Sea Urchin Feeding Mechanisms

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Abstract. Selective particle-picking mechanisms of clypeasteroid echinoids (sand dollars and related taxa) are well-known. Those of the extant outgroup to clypeasteroids, the cassiduloids (lamp urchins), have not been analyzed to determine the origins of this sophisticated feeding mechanism. *Cassidulus caribaeorum* Lamarck, 1801, is a small cassiduloid living in the coarse, carbonate sands of protected beaches. The total gut contents of 24 specimens of *C. caribaeorum*, representing a full size range, were studied. The distribution of particle sizes in this sample was not significantly different from that of beach sediment, indicating that *C. caribaeorum* is probably not a selective deposit feeder. Juveniles with a test length of less than 3.5 mm do not feed, but all echinoids that are at least 5 mm long have full, or almost full, guts. The size of the mouth does not limit the sizes of particles eaten, regardless of the size of the animal. Allometric analyses suggest that podial size is also not a strong predictor of ingested particle size. *In vivo* and histological observations differentiate between the test cleansing functions of the spines and ciliary currents and the feeding activities of specialized podia. The new role played by accessory podia in food-collection by *C. caribaeorum* is a synapomorphy for cassiduloids and clypeasteroids, whereas the exclusive use of phyllopodia seen in earlier irregular echinoids is plesiomorphic.

Introduction

The mechanism of particle collection by deposit-feeding members of the Clypeasteroida (sand dollars, key-

hole urchins, sea biscuits, and allies) has been well studied in recent years (Ellers and Telford, 1984; Telford *et al.*, 1985, 1987; Telford and Mooi, 1986) and is well enough understood that a stochastic model has been developed and used to provide a computer simulation of the process (Telford, 1990). Particles are drawn from the sediment one by one, by foraging podia (or tube feet), and are then passed from podium to podium to reach the food grooves (shallow, ditch-like depressions in the test surface that lead from the distal regions of the test to the mouth). Stubby podia within the grooves then direct the material, which is usually gathered into a mucous cord, to the mouth. Although the collection of particulate material by suckered podia occurs in other echinoid groups, no research has specifically addressed the evolution of the mechanism.

Cassiduloids (lamp urchins) are more closely related to the Clypeasteroida than to any other extant echinoid group (Mooi, 1990a; Suter, 1994), and would therefore be the most suitable outgroup for a comparative and evolutionary study of clypeasteroid feeding mechanics. Of the 30 or so living cassiduloid species, 5 occur in American waters (Mooi, 1990b). Only a single recent paper (Gladfelter, 1978) has described the biology of the commonest American cassiduloid, *Cassidulus caribaeorum* Lamarck, 1801. The feeding mechanisms in other cassiduloids have only been cursorily investigated. For example, Thum and Allen (1976) suggested that both "oral podia" and circumoral bourrelet spines lift particles into the mouth of *Echinolampas crassa*. Some selectivity was implied by their comments that larger particles were excluded from the diet because of the fixed size of the peristome, and that smaller particles were not eaten because the echinoid had difficulty manipulating them. Mooi

Received 27 November 1995; accepted 16 July 1996.

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(1990b) summarized what little is known of the biology of cassiduloids, showing that a comprehensive picture of feeding biology in the group awaits further study.

In this paper we describe the gross morphology and histology of every structure involved in feeding, thereby distinguishing them from the structures used in cleansing of the test. In doing so, we provide the only detailed description of all spine types and nonrespiratory podia found on a cassiduloid. We also perform the first allometric study of podia in an echinoid, concentrating on those podia involved in food-gathering and handling. On the basis of these observations, we describe the feeding mechanism of *Cassidulus* and contrast it with that of clypeasteroids. A detailed analysis of ingested material includes a statistical test for selectivity by this deposit-feeding urchin. Finally, we synthesize all these new data in a discussion of the evolution of phyllopodial feeding within the extant Irregularia (Holectypoida, Spatangoida, Holasteroida, Cassiduloida, Clypeasteroida) and an exploration of the origins of the sophisticated podial particle-picking mechanism (PPPM) in the scutelline sand dollars.

Materials and Methods

Specimen collection

Specimens of *C. caribaeorum* were collected at Loblolly Bay, on the resort island of Anegada, British Virgin Islands. The urchins occur 10–100 mm beneath the surface of the sediment in a small berm at the water's edge, at depths ranging from 0.5 to 1 m. They were collected by hand and transported live to a temporary laboratory for *in vivo* observation. Other specimens were fixed for 24 h in 2% glutaraldehyde in seawater, and stored in 70% ethanol. Specimens for podial measurements were first relaxed by dropwise addition of ethanol until no response to touch was observed.

In vivo observations

Feeding was observed by viewing specimens with a stereomicroscope focused on an inclined, front-silvered mirror mounted at 45° beneath a small aquarium. The aquarium floor was sparsely covered with native sand from Loblolly Bay. Ciliary currents were visualized from both oral (bottom) and aboral (top) surfaces. A suspension of India ink or finely ground carmine particles in seawater was introduced between the spines *via* a hypodermic syringe, using the methods of Telford *et al.* (1985). On the aboral surface, these suspensions were added to areas around the apical system. On the oral surface, they were placed in areas adjacent to the mouth, and just below the ambitus (the edge of the test).

Histology

Specimens were preserved in 10% formalin in seawater, or 2% glutaraldehyde in seawater passed through a Millipore filter. The following regions of the test and associated appendages were then sampled: ambulacrum I adjacent to the peristome, ambulacrum III on the oral surface, the locomotory area in ambulacrum 1, the naked zone in interambulacrum 5, and the anal sulcus (the deep groove in the test leading from the periproct, or anal region, to the posterior edge of the test). Using the methods outlined in Mooi (1986a), we decalcified the samples and then embedded them in epon araldite. Blocks were then sectioned at 0.5–1.0 μm intervals and the sections affixed to microscope slides in series. The epon araldite was removed in a saturated solution of sodium hydroxide in 95% ethanol, and stained with thionin, toluidine blue, or Milligan's trichrome according to techniques described in Humason (1962). Sections were observed with a compound microscope and drawn with the aid of a camera lucida. Additional information about the morphology of spines and the positions and lengths of ciliary bands was gathered by observing "living" spines that had just been removed from the test and placed in seawater on well slides, or by slightly staining whole mounts of preserved spines with toluidine blue.

Test and podial measurements

Test length, width, and height, and peristome length and width were measured to the nearest 0.1 mm with electronic calipers. The diameters of podial suckers on preserved specimens were measured with a calibrated eyepiece micrometer. Before the podia could be measured, they had to be made more visible, and this was accomplished by briefly staining the entire urchin in 1% methylene blue. For ambulacra III, IV, and V, every podium that was visible while looking directly at the oral surface was measured. When podia in ambulacra IV or V (called the paired ambulacra because they are almost mirror images of ambulacra II and I, respectively) were missing or obviously regenerating, measurements were obtained from the corresponding podia in the ambulacra on the other half of the test.

Sediment and gut content analyses

Five samples of subsurface sediment were collected by hand from among the population of *C. caribaeorum* in the small berm at Loblolly Bay described above. Care was taken to collect the sediment samples from regions in the berm that contained the highest concentration of urchins. Small waves lapping against the berm kept the sediment constantly stirred, and stratification within the berm was not observed. Particles were strewn on glass slides and examined under a compound microscope

equipped with a camera lucida. A digitizing tablet (Houston Instrument Hipad) was positioned adjacent to the microscope for simultaneous viewing. For each particle of sediment, the two longest dimensions orthogonal to each other were obtained by digitizing. The mean dimension was calculated, and a computer program sorted the particles and assigned them to size classes at 100- μ m intervals (Telford, 1990). Twenty-four specimens ranging from 3.7 to 26.8 mm were dissected and their gut contents removed for individual analyses. The number of particles in the gut of each individual was counted under a dissecting microscope, and every particle was digitized as described above.

Statistical procedures

All regressions were calculated by least squares fit of log-transformed data. An exponent (b) of 1.00 therefore indicates isometry of the two variables, and the coefficient (A) is the value of the dependent variable when the independent variable is 1.00.

The significance of the differences between the frequency distributions of particles from urchin guts and Loblolly Bay sand was tested using a log-likelihood ratio test (G -statistic) (Sokal and Rohlf, 1981):

$$G = 2 \sum O_i \ln (O_i/E_i)$$

where O_i is the observed frequency in the i th class and E_i the expected frequency. The null hypothesis, H_0 , states that there is no difference between gut contents and surrounding sediment. The E_i values were obtained by multiplying the total number of particles in each gut sample by the proportions, p_i , of the particles in the surrounding sediment.

A stochastic algorithm was written to generate simulations of gut contents based on a random selection of particles. The probability of a foraging podium encountering a particle of any given size class depends on the contribution which that size class makes to the planar area of the sediment (Telford, 1990). This is estimated as:

$$A_i = (O_i * (M_i)^2) / (\sum O_i * (M_i)^2)$$

where A_i is the contribution to planar (or projected) area, O_i is the particle frequency, and M_i the mean particle dimension in the i th size class. Numbers between 1 and 100,000 were assigned successively to the particle size classes, according to their proportional representation. To simulate gut contents, random numbers were generated (seeded on the computer clock) and particles were added to the corresponding size classes. For each specimen, the observed number of particles in the gut determined the number of random particle selections to be made. Ten replicates were run for each specimen and for Loblolly Bay sand, and the means and SD were calcu-

lated. Frequency distributions were tested by the G -statistic. In a process analogous to bootstrapping, 10,000 random simulations of the pooled gut contents were made, and the distribution of the resulting G -statistics was examined.

Results

Podial morphology

Four types of podia are found in the ambulacra of *C. caribaeorum*: respiratory podia, buccal podia, phyllopodia, and accessory podia. Respiratories are flattened, leaflike podia mounted on paired pores that continue to the apical system in two rows. These podia have very thin walls with reduced musculature. They lack attachment disks, and do not function as manipulatory structures. Respiratory podia are not involved in feeding and so are not treated here, except to note modifications of ciliary currents that enhance their efficiency in gas exchange (below). The other three types of podia—nonrespiratory—are well-muscled, thick-walled tubes terminating in a distinct terminal disk. Figure 1 illustrates the general appearance and histology of two of these three types. Each of these podia is mounted on a single pore that passes through the test to an internal saclike ampulla which is, in turn, connected to the lateral and radial water vessels in the typical echinoid manner. There is generally one podium per plate in the ambulacra. However, one plate in each pair of ambulacral basicoronals bears two podia: a buccal and a phyllopodium.

Each ambulacrum has a pair of buccal podia. Buccals are the first podia in the ambulacral series, and are located adjacent to the peristome, one in each ambulacral basicoronal plate. They are also the first podia to appear during development after the primordial podia (the latter comprise the "terminal tentacles" in the ocular plates). Even in adults, buccals are not very extensible (Fig. 1B), and are seldom capable of reaching more than a millimeter from the surface of the test. Frequently exceeding 300 μ m in diameter in adults (Fig. 1C), the terminal disk is greatly expanded and more than twice the diameter of the stem when the podium is extended.

Phyllopodia can also exceed 300 μ m in disk diameter, but tend to be somewhat smaller than the buccals in a given individual. Phyllopodia are very similar to buccals in having expanded disks, but are capable of greater extension. In each ambulacrum, there are two slightly curved arcs of phyllopodia which together form a structure known as the phyllode. In adults, each arc of the phyllode, composed of 5 or 6 phyllopodia, continues distally from a corresponding buccal podium (Fig. 1). The number of phyllopodia increases with size of the individual echinoid, and the podia tend to decrease in disk diameter with distance from the peristome.

Distal to the phyllodes are the accessory podia. These

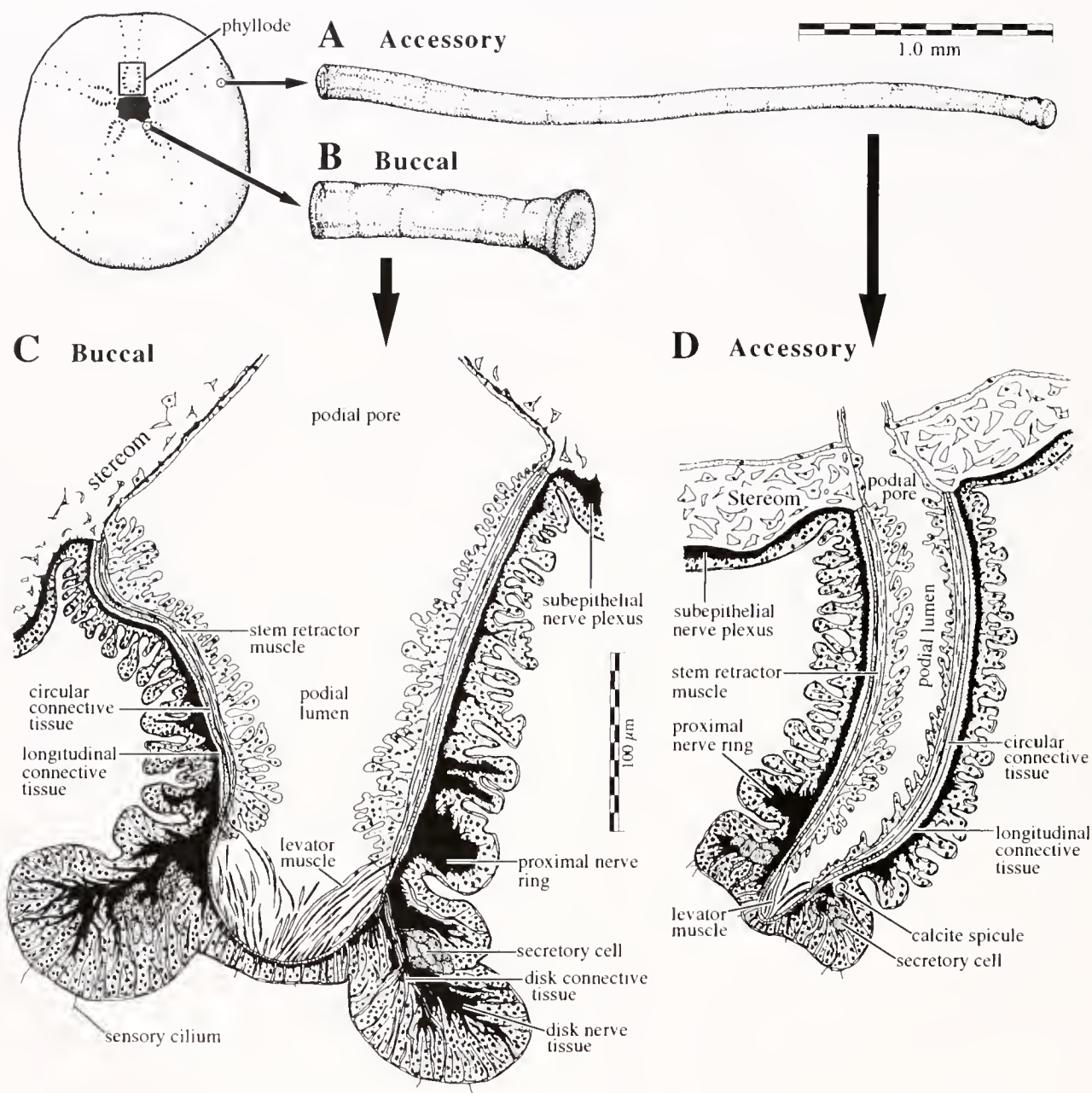


Figure 1. Podia of *Cassidulus caribaeorum*. (A) Appearance in life of fully extended accessory podium. (B) Appearance in life of fully extended buccal podium. (C) Longitudinal section showing histology of retracted buccal podium. (D) Longitudinal section showing histology of retracted accessory podium. View of oral surface in upper left indicates locations of podia in A–D. In C and D, the scale bar is corrected for tissue shrinkage during histological preparation.

are highly extensible, with slender stems that can reach more than 3 mm beyond the test surface (Fig. 1A). The diameter of the disk (seldom exceeding 150 µm) is not much greater than that of the shaft, even when the accessory podium is fully extended and the wrinkles in the epithelium (Fig. 1D) are smoothed out. A single accessory occupies each ambulacral plate, making two rows per ambulacrum until they give way to the closely spaced

respiratory podia that constitute the lanceolate, gill-like structures on the aboral surface known as the petaloids.

Podial histology

The anatomy and histology of the buccal and accessory podia are summarized in Figure 1C and D. The histology of phyllopodia is very similar to that of buccal po-

dia, so they are not figured. The water vascular system extends into the stem to form a coelomic podial lumen lined with epithelium. There is a series of stem retractor muscles between this epithelium and a connective tissue sheath. These retractors insert around the outer rim of the podial pore and run up the shaft inside the connective tissue sheath to a point just proximal to the blind end of the sheath. The sheath consists of longitudinal and circular collagenous elements that stained vivid green in Milligan's trichrome; the fast green in this trichrome stain is specific for connective tissue. Inside the distal part of the connective tissue sheath is a series of levator muscles, each fiber of which arises from the inside of the blind end of the sheath and inserts near the point at which the stem retractors end. Just outside the connective tissue sheath is a subepithelial nerve plexus confluent with that overlying the test. The plexus thickens to form a nerve ring just proximal to the disk, and also gives rise to ramifications in the epithelium of the disk itself. The disk is made up of greatly thickened epithelium containing both ciliated and unciliated cells, secretory cells, and fibers of connective tissue arising from the connective tissue sheath. The central part of the disk supports short cilia, and the more peripheral parts of the disk tend to bear somewhat longer cilia. Within the epithelium of the central part of the disk are support cells bearing internal filaments that stain red in Milligan's trichrome. The acid fuchsin usually indicates the presence of muscle fibers, but in this case the red color seems to be resident in tonofilament bundles in the support cells (*sensu* Flam-mang and Jangoux, 1994). There is an abundance of secretory tissue in the peripheral parts of the disk epithelium, most of which stains purplish or dark blue in thionin and toluidine blue. The histological procedures used here were sufficient only to reveal the presence of secretory cells, but not the intracellular features that distinguish the various types.

The histology of all three types of nonrespiratory podia is very similar. The buccals and phyllopodia are the most alike, but the accessories tend to have fewer levator muscles, a narrower podial lumen, and much smaller disk and associated structures. Calcareous elements, when they are present at all, are found only in the accessory podia (Fig. 1D), never in phyllopodia or buccals. These spicules are small, rod-shaped elements embedded in the outer layers of the connective tissue sheath at the proximal part of the disk near the point at which the stem retractors end. Even in the accessory podia, spicules are extremely rare and when present, there is usually only one in any given podium.

Spine morphology and ciliation

Two general types of spine can be found on the test of *C. caribaeum*. Primary spines (*sensu* Mooi, 1986c) are

the longer type, occupying the largest tubercles on both aboral and oral surfaces. Miliary spines occupy the smaller tubercles interspersed among the primary tubercles. We distinguish among four major types of primary and four types of miliary spines in *C. caribaeum* based on dimensions and curvature of the shaft, ciliary pattern, and position on the test (Fig. 2).

The longest spines on the top of the echinoid are known as aboral and anal sulcus primary spines (Fig. 2A and C respectively). The aboral primaries are slightly swollen distally, and distributed evenly over the aboral surface to form a layer of almost constant depth. Aboral primaries are generally slightly longer than the anal sulcus spines. The latter are known only from the anal sulcus, a shallow trough that extends posteriorly from the periproct (Fig. 2). On both these primary types, two ciliary bands run from the base of the spine distally for about 75% of the spine's length. As discussed below, the ciliary bands are located on opposite sides of the shaft in the pattern described by Mooi (1986c). Aboral and anal sulcus miliaries are found in the same areas (respectively) from which aboral and anal sulcus primaries are known. In each case, they are only about 75% the length of their corresponding primary spines, but the ciliary bands extend all the way to their ends (Fig. 2B, D). The miliary spines of some scutelline clypeasteroids terminate in balloonlike sacs consisting of swellings covered by epithelium and filled with collagen fibers (Mooi, 1986c), but the miliaries of *C. caribaeum* lack distal swellings of any kind. All the spines on the aboral surface are slightly bent where the shaft meets the base so that the spine leans at a slight angle downslope from the apex of the test.

On the oral surface, there are two types of primary spines. Locomotory spines occupy two broad locomotory areas, one on each side of the oral surface near the edge of the test (Fig. 2). Each locomotory spine has a strong bend where the shaft meets the base so that the spine bends towards the midline of the oral surface. Ciliary bands extend from the base for about 20% of the length of the spines (Fig. 2E). Locomotories are the longest spines on the echinoid, occasionally exceeding 3 mm in length on adults more than 25 mm in test length. However, these spines vary slightly in length, with the longest in the centers of the locomotory areas and the shortest at the peripheries of the areas. Interspersed among the locomotories are very short miliaries that are among the shortest spines on a given specimen (Fig. 2F). Like the aboral miliaries, ciliary bands run almost the entire length of the spine. There are no primary spines in the so-called naked zone, which is populated only by sparsely distributed miliary spines that are similar in ciliation to, but slightly longer and more robust than, those in the locomotory area (Fig. 2G). In *C. caribaeum*, the slight bulging of the interambulacral basicoronal plates intrudes into the peristomial region cre-

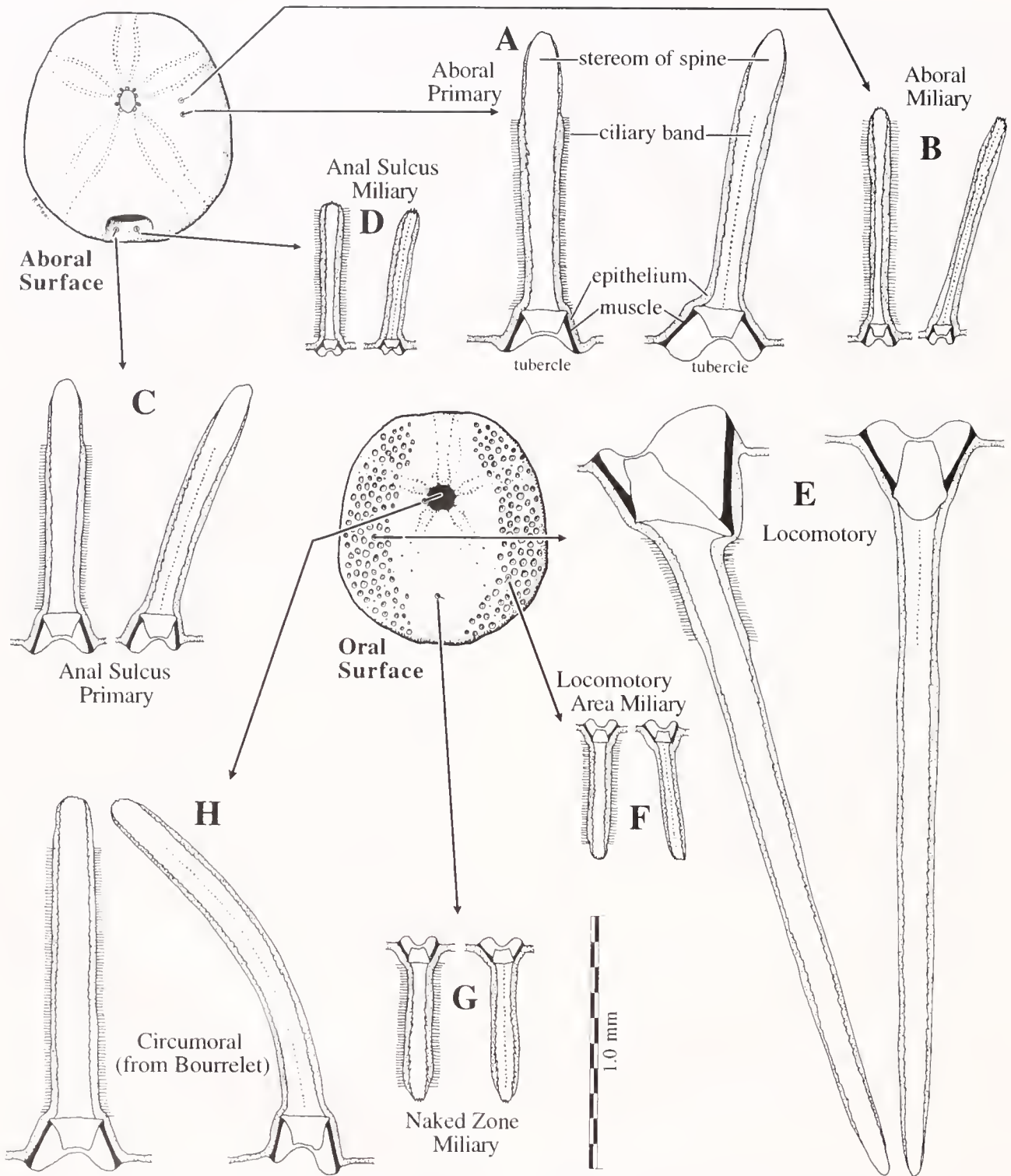


Figure 2. Morphology and ciliation of spines of *Cassidulus caribaeorum*. Arrows indicate site of origin for each spine type: A–D from aboral surface; E–H from oral surface. For A–H, each pair of drawings shows two orthogonal views of same spine. Shading conventions used for labeled structures in A apply to all drawings in A–H. Scale bar refers to all spines in A–H. On drawing of oral surface, naked zone is outlined by fine, dotted lines.

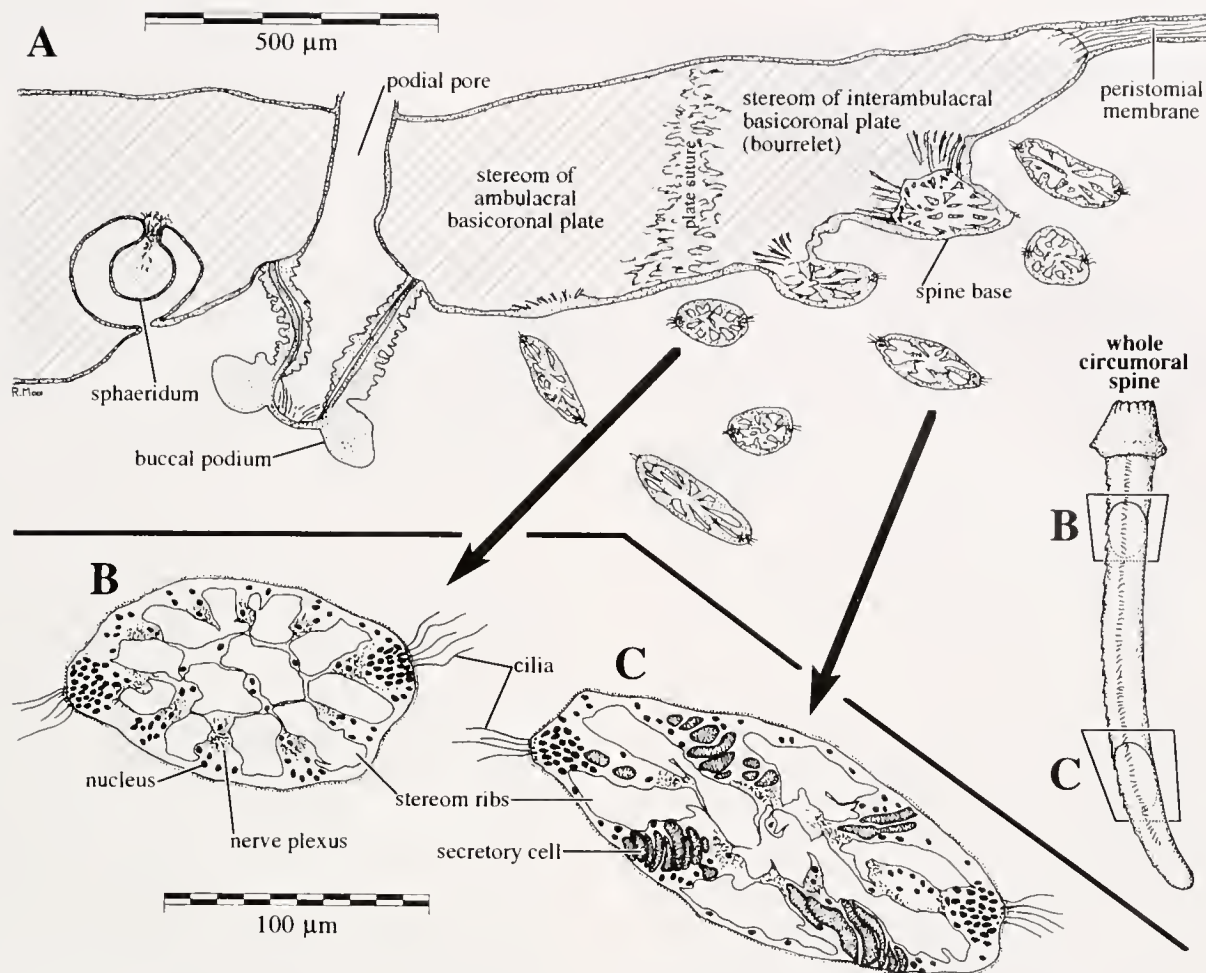


Figure 3. Histology near peristome of *Cassidulus caribaeorum*. (A) Radial section of peristomial area adjacent to, but not through, perradial suture of ambulacrum I. Plate suture is between ambulacrum and interambulacrum. (B) Slightly oblique cross-section through proximal part of circumoral spine. (C) Slightly oblique cross-section through distal part of circumoral spine. Sections in B and C correspond to those shown in whole circumoral spine on right.

ating a “bourrelet.” On these bourrelets are series of densely packed, strongly curved circumoral spines (Fig. 3A) that arch over the peristomial area. When moved towards the mouth, these spines cover almost all of the peristome except for small areas in the adjacent ambulacra, but can be flexed outwards from the peristome to admit larger food particles. These spines have the longest ciliary bands on the urchin. The spines themselves can be longer than 1 millimeter, and the bands stop short of the tip (Fig. 2H).

Spine histology

Spines are made up of a stereom shaft extending distally from the base. The base articulates on a tubercle to form the ball-and-socket joint typical of most echinoids. A ring of muscles surrounds the base so that the spine is free to swing in any direction. The shaft is made of be-

tween 6 (in miliaries) and more than 15 (in larger primaries) parallel ribs assembled in a cylinder and connected by a meshwork of struts between and internal to the ribs. Each spine is covered by a thin epithelium below which lie thin strips of nerve tissue between the stereom ribs of the shaft. These strips constitute the subepithelial nerve plexus of the spine (Fig. 3B, C). On diametrically opposing sides of the spine are two ciliary bands. The cells in these bands each support a single cilium that protrudes 20 or 30 μm beyond the level of the cuticle. The ciliary bands are 4 or 5 cells wide and create a slightly thickened, longitudinal ridge of epithelium (Fig. 3B, C). Most spines, whether primary or miliary, bear masses of conspicuous secretory cells between the stereom ribs. These stain brilliant purple in toluidine blue, and have a distinct granular appearance (Fig. 3C). The cells are absent from the proximal region of the spine (Fig. 3B), but increase in number towards the tip.

Ciliary currents

Currents generated by the ciliary bands on the spines flow between the spine shafts in a pattern common to all the specimens observed (Fig. 4). Fresh seawater can enter the spaces between the spines anywhere except places at which flow leaves the test, but the primary entry points of ambient water seem to be at the center of the aboral surface (Fig. 4A). Centrifugal flow then continues downward towards the periphery of the test (ambitus). Currents flowing down the centers of the ambulacra flow circumferentially for a short distance until they leave the petaloids by passing between the respiratory podia. Particularly strong flow is associated with the anal sulcus area, at which currents sweep across the periproctal plates and then along the sulcus to the ambitus. Some flow away from the test was observed at the edge of the hooded region just above the periproct, and at the ambitus below the periproct (Fig. 4A). However, the vast majority of the flow continues around the ambitus and onto the oral surface.

Anteriorly on the oral surface, the currents flow centripetally until they reach the mouth; then they converge and flow downwards away from the center of the oral surface. Posteriorly, the currents also converge, this time at a point in the posterior region of the naked zone. This area of convergence is characterized by additional downward flow away from the test. Strong currents of water that did not leave the echinoid at the peristomial region of convergence were seen to travel on either side of the peristome and then along the center of the naked zone directly towards the posterior region of convergence (Fig. 4B). Ciliary currents were never observed to augment the feeding process, and were in fact very active in com-

pletely removing particulate material from the test surface, and in providing continuous current over the respiratory podia of the petaloids.

Feeding mechanism

C. caribaeorum feeds exclusively with its podia; spines and pedicellariae were never seen to aid in food-gathering or ingestion. Accessory podia first probed and then attached to particles of ambient sediment. There was no obvious selection for particular elements of the sediment, in spite of the abundance of bright pink or discoidal foraminifera. Phyllopodia frequently procured particles for ingestion, but the task of gathering food particles was clearly divided between accessory podia and phyllopodia. Although the accessories on the oral surface were more active in food-gathering, those on the aboral surface were also seen to draw in and pass particles around the ambitus to podia on the oral surface. A given particle was then manipulated towards the peristome, frequently by more than one podium at a time, until the phyllopodia and buccal podia could move the particle into the mouth. Once picked up by podia, particles were not agglutinated in masses or strings. Instead, each particle was handled separately until ingested. The circumoral spines moved downwards away from the peristome to allow passage of larger particles into the mouth. The mouth opening itself could be greatly enlarged through retraction of the peristomial membrane towards the peristomial edge, thereby allowing the particles to be swallowed. It was not clear how the act of swallowing was actually accomplished, although the peristomial membrane was equipped with liplike regions directly adjacent to the

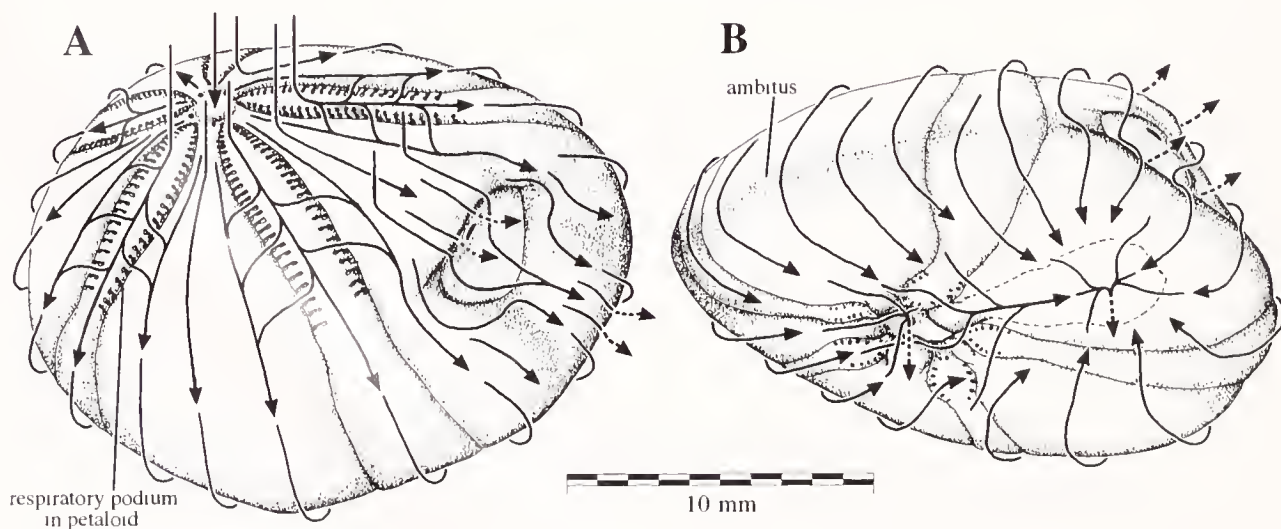


Figure 4. Ciliary currents on *Cassidulus caribaeorum*. (A) Oblique aboral view from the posterior. (B) Oblique oral view from the posterior. Spines omitted for clarity. Solid arrows indicate flow towards test or within spine field; dashed arrows indicate flow away from test.

mouth that could aid in pushing the particles up into the esophagus as the mouth was drawn shut.

Particle analysis of Loblolly Bay sediment

The sediment of Loblolly Bay is a clean, calcareous sand of biogenic origin, consisting of about 30% coral fragments, 15% foraminifera, 15% mollusc shells, 5% echinoderm bits, 5% coralline algae, and 30% unidentifiable debris. Table I shows the distribution of different particle sizes, and their collective contributions to planar area and volume. For each size class the contribution to area is the sum of the squares of the mean dimensions expressed as a percentage of the total. Similarly, the volume contribution is based on the cubes of the mean linear dimensions.

Particle analysis of gut contents

When compared to predicted frequencies, particle frequency distributions in the gut contents of individual urchins differed significantly. However, no consistent pattern of variation was detectable, so the data do not point to particle size selection. The particle size frequency distributions for gut contents of 24 individuals ranging from 3.7 mm to 26.8 mm test length are shown in Table II. These data indicate that all urchins feed on similarly sized particles, and that the population as a whole is not selecting for any given particle size class or classes. *G*-statistics were calculated to test the significance of the observed and expected distribution of particle size frequencies in the pooled gut contents (Table III). The size classes below 500 μm were represented in the gut samples by observed frequencies of less than 100. They were

pooled to avoid inflation of the *G*-statistic. Similarly, particles greater than 1600 μm (1610.8–1987.4 μm) were pooled to make the test as conservative as possible. *G*-statistics were calculated for expected frequencies based on simple particle frequency proportions ($G = 6427.245$, $df = 12$), on contribution to area ($G = 141.583$, $df = 12$), and on contribution to volume ($G = 1984.638$, $df = 12$). All of the *G*-statistics are significant at the 0.01 level of probability ($G = 26.217$).

Although statistically significant, the magnitude of the difference in *G* values suggests that particles are most probably collected according to the contribution made to area by each size class. The probability of getting a *G* value of this magnitude was tested by the stochastic simulation of 10,000 samples consisting of 20,514 particles. The range was 16.926 to 241.701 and the mean *G* for these simulations was 103.223 (± 44.714), the median was 110.206, and the 95% confidence limits were 35.161 to 147.903. Among the 10,000 determinations of *G*, there were only 63 lower than 26.217, thus 99.37% of them would be ranked as "statistically significant."

Test allometry

Table IV presents the results of regression analyses for various test dimensions, using test length along the antero-posterior axis as the independent variable. The exponents for test width and test height do not differ significantly from 1.00, indicating that *C. caribaeorum* does not change shape as its size increases. In contrast, the exponents for width and length of the peristome indicate that the aperture increases more slowly than test length, and thus the opening becomes relatively smaller as the urchins increase in size.

Podial allometry

Results of regression analyses of podial disk diameters (dependent variables) and test length (independent variable) are shown in Figure 5. Separate analyses for the podia in ambulacra III, IV, and V showed nonsignificant differences, so the data were pooled. All exponents are significantly less than 1.00: as the test size increases, the podial disk diameter becomes relatively smaller. Within the observed size range of *C. caribaeorum* (up to 26.8 mm test length), the disks of the buccal podia are always slightly larger than those of the phyllopodia, but the two structures are otherwise indistinguishable. Although the accessory podia are similar in general morphology to the buccals and phyllopodia, they have markedly smaller disks. Moreover, this disparity in disk size becomes increasingly apparent as the urchin becomes larger.

Gut content allometry

Allometric analyses were performed on the numbers of particles in the gut, and on the mass of the gut contents

Table I

Frequency distribution of different sized particles in sand from Loblolly Bay (Anegada, B.V.I.), collected from small berm inhabited by *Cassidulus caribaeorum*. Data represent 5 pooled samples

Particle size class, μm	<i>n</i>	Proportion by frequency	Proportion by area	Proportion by volume
<500	1363	0.12615	0.01356	0.00391
500–600	458	0.04239	0.02205	0.01498
600–700	2715	0.25126	0.18257	0.13684
700–800	2601	0.24072	0.23286	0.20248
800–900	1766	0.16344	0.20308	0.19138
900–1000	980	0.09070	0.14078	0.15431
1000–1100	491	0.04544	0.08616	0.10432
1100–1200	174	0.01610	0.03663	0.04867
1200–1300	89	0.00824	0.02213	0.03291
1300–1400	69	0.00639	0.02001	0.03227
1400–1500	35	0.00324	0.01171	0.01968
1500–1600	26	0.00241	0.00994	0.01887
>1600	38	0.00352	0.01852	0.03838
Totals	10805	1.00000	1.00000	1.00000

Table II

Particle size frequency in the guts of 24 *Cassidulus caribaeorum* arranged in order of test length (TL)

TL (mm)	Particle size class ($\times 100 \mu\text{m}$)													Total
	<5	>5	>6	>7	>8	>9	>10	>11	>12	>13	>14	>15	>16	
3.7	0	0	1	0	0	0	0	0	0	0	0	0	0	1
4.3	0	0	0	3	0	0	0	0	0	0	0	0	0	3
4.3	0	1	1	5	14	9	1	2	1	0	0	0	0	34
4.9	1	8	11	19	16	6	3	2	1	0	0	0	0	67
5.4	1	10	23	23	12	7	0	0	0	0	0	0	0	76
6.7	8	28	38	38	13	10	2	0	0	0	0	0	0	137
7.0	3	4	48	47	24	17	5	2	1	1	5	9	6	172
8.3	5	26	77	59	40	10	4	7	3	0	0	0	0	231
9.2	6	27	58	72	65	63	31	14	2	1	0	0	0	339
9.3	2	31	101	77	69	40	15	10	3	2	1	1	0	352
11.3	8	18	99	115	79	65	27	14	3	2	2	11	10	453
11.3	14	8	125	131	98	47	33	16	5	24	14	13	21	549
12.3	15	39	151	150	98	68	47	17	11	18	4	11	10	639
13.1	31	25	188	191	105	42	28	13	23	23	12	12	11	704
13.4	13	14	152	185	139	83	30	16	8	27	25	13	1	706
15.2	8	22	234	260	238	108	64	27	14	9	16	12	31	1043
15.6	0	11	119	198	215	177	94	38	23	17	8	2	0	902
15.8	3	0	192	213	222	179	97	49	19	9	5	31	21	1040
17.3	5	33	137	273	303	244	164	71	49	27	17	9	1	1333
18.5	17	4	176	328	332	270	179	99	48	74	15	8	2	1552
20.1	22	26	434	507	362	236	274	79	52	37	23	12	7	2071
20.9	58	65	516	658	473	447	184	77	59	42	28	27	37	2671
22.6	34	49	513	652	620	414	239	46	51	36	21	13	8	2696
26.8	27	8	390	620	671	370	266	159	82	66	47	22	15	2743
Total	281	457	3784	4824	4208	2916	1787	758	458	415	243	206	181	20514

Table III

Observed and expected frequencies of different sized particles (with G-statistics) in the pooled gut contents of 24 *Cassidulus caribaeorum* shown individually in Table II

Midpoint of size class (μm)	Observed F	Expected F	Expected A	Expected V
250	281	2588	278	80
550	457	870	452	307
650	3784	5155	3746	2808
750	4824	4938	4776	4164
850	4208	3353	4166	3936
950	2916	1861	2889	3169
1050	1787	932	1767	2140
1150	758	330	751	998
1250	458	169	454	675
1350	415	131	410	659
1450	243	66	240	404
1550	206	49	204	387
1750	181	72	380	787
G (12 df)		6427.245	141.583	1984.638
P		≤ 0.001	< 0.001	≤ 0.001

Expected frequencies were calculated assuming random collection by these urchins using the probabilities of encountering the particles based on their frequency (F), and their contributions to planar surface area (A) and volume (V) of the surrounding sediment.

(dependent variables), and test length (independent variable). The results are shown in Table IV.

Discussion

Roles of the external appendages in feeding and cleansing activities

As in clypeasteroids, the podia of cassiduloids are clearly the "right equipment" for food-gathering (see arguments in Telford *et al.*, 1985, and Mooi, 1986b). The histology of cassiduloid podia is very similar to that of clypeasteroids, with well-developed sensory capabilities, secretory cells for provision of adhesive substances, and musculature for efficient particle manipulation toward the mouth (Fig. 1). However, cassiduloid podia tend to be larger than those of clypeasteroids, even in small forms such as *C. caribaeorum*. This is especially true for the greatly expanded tips of the phyllopodia found on cassiduloids. Our observations of gross morphology suggest that these are more reminiscent of those in regular echinoids, in which the disk diameter to shaft diameter ratio is about 2:1. In clypeasteroids, this ratio is closer to 1:1 (Mooi, 1986a). Clypeasteroids also have a greater diversity of podial types and a much higher total number of podia than cassiduloids. These additional differences

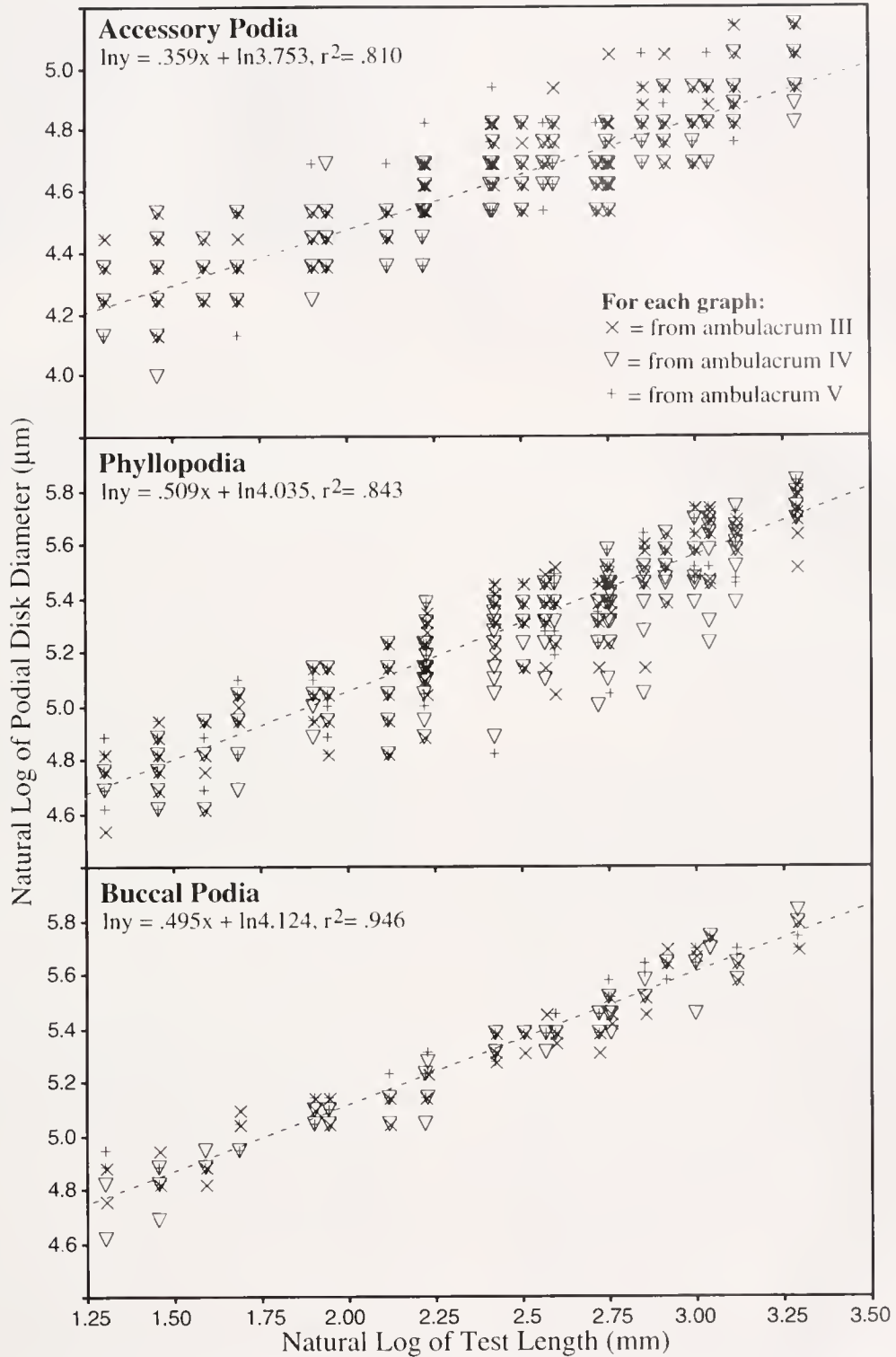


Figure 5. Plots and regression analyses of podial disk diameter against test length for each of the three nonrespiratory podial types in 24 specimens of *Cassidulus caribaeum*.

are related to a radically different manner of feeding, and to differences in the size ranges of sediment particles that form the diets of these two echinoid groups. Clypeaster-

oids handle many thousands of particles at any moment during the feeding process, employing similar numbers of greatly miniaturized accessory podia to collect these

Table IV

Regression analyses of test dimensions and gut contents (dependent variables) against test length (independent variable, mm) in *Cassidulus caribaeorum* ($n = 20$)

Dependent variable	Exponent (b)	Coefficient (a)	r^2
Width (mm)	0.97	0.925	0.996
Height (mm)	1.04	0.404	0.988
Peristome width (mm)	0.56	0.530	0.927
Peristome length (mm)	0.54	0.434	0.959
Number of particles in gut	2.34	1.69	0.986
Mass of particles in gut (g)	2.71	0.002	0.982

particles (see evolutionary discussion below). Banks of podia of differing dimensions in different regions of the test work together to pass particles into food grooves where they are amalgamated into loose strings by the secretory and manipulatory action of specialized podia lining the grooves. Cassiduloids generally feed on coarser sands than clypeasteroids, and the entire complement of cassiduloid podia is capable of picking up only a few particles at a time. Cassiduloids lack food grooves, relying instead on enlarged phyllopodia that manipulate individual particles towards the mouth.

Histology and *in vivo* observations (Figs. 2, 4) of other external appendages of *C. caribaeorum* indicate that only the podia are used in food-gathering. This is completely consistent with what is known about feeding in other irregular echinoids. Although spines were once thought to function in feeding of clypeasteroids, this has been shown not to be the case (Telford *et al.*, 1985; Mooi, 1986b, c). Cassiduloid spines are likewise not employed in feeding. Even the circumoral bourrelet spines do not aid in the ingestion of particles, although they might help in keeping particles next to the peristomial membrane until they can be swallowed.

The easily observed, powerful ciliary currents of *C. caribaeorum* are strictly for cleaning and ventilation. In burrowers such as this species, the importance of ciliary flow cannot be overestimated. The hydrodynamic principle of continuity is evident in the way that fresh seawater is continuously introduced, largely at the apical region, to flow down around the test and then leave it in specific areas at the posterior and oral regions (Fig. 4). This principle plays an important role in keeping the test surface free of obstructing fine material and in meeting the need of the epithelium for dissolved organic nutrients and gas exchange. In addition, surface flow produced by cilia on the aboral primary and miliary spines is instrumental in maintaining flow over the respiratory podia in the petaloids (Fig. 4). Gladfelter (1978) noted the high respiratory rates of *C. caribaeorum*. We also noted that even slight drops in the flow of fresh seawater through the

sediment in which the echinoids were burrowed induced them to come to the surface immediately. Both of these observations suggest the importance of ciliary currents in alleviating such stress in the wild. Spines on both surfaces of *C. caribaeorum* are also capable of producing mucous secretions (Fig. 3), but their purpose remains obscure, except that a general cleansing function, such as that seen in spatangoids and some members of the clypeasteroid genus *Clypeaster*, seems reasonable (Telford *et al.*, 1987).

The allometry of particle collection

The allometric analysis of test dimensions indicates that as size increases in *C. caribaeorum*, the proportions of the test do not change. In contrast, podial diameters increase more slowly than test dimensions, so that the podia of adult urchins are relatively much smaller than those of juveniles. *C. caribaeorum* is a brooding urchin in which juveniles (less than 4 or 5 mm test length) are retained among the aboral spines of the adult females. When the juveniles are eventually released, they inhabit exactly the same sediment as the adults, feeding on the same array of particle sizes. The similarity in podial dimensions in different-sized urchins should not, therefore, be surprising. The size of the peristomial opening becomes relatively smaller as urchins increase in size, and this too should be expected if the urchins are ingesting similar-sized particles.

Comparisons of gut contents in urchins of different sizes show that they do, in fact, feed on similarly sized particles (Table II). No consistent differences were found in the linear dimensions or in the masses of particles in different sized urchins. Allometric analysis (Table IV) showed that the mass of material in the gut increases less rapidly than the cube of the linear dimension (b values of 2.34 for number of particles and 2.71 for mass of particles vs. test length is significantly different from 3, $P = 0.005$), suggesting either that the gut in *C. caribaeorum* does not scale isometrically with body volume, or that the animals do not keep the gut full at all times.

Gladfelter (1978) considered *C. caribaeorum* to be a somewhat selective feeder. Selectivity by deposit-feeding organisms results in a consistent, statistically significant, difference between the particle size frequency distributions of ingested material and of the surrounding sediment. This may arise passively from constraints in the collection mechanism, as it does in sand dollars (Telford, 1990), or as a consequence of active sorting and selection, as in some tentaculate worms (Jumars *et al.*, 1982). Most data on deposit feeding come from sieve analyses, in which the mass of material in each size class in the gut is compared to the distribution in the sediment. The unstated underlying assumption is that particles are available in proportion to their mass representation in

the sediment. In a stochastic simulation of deposit feeding in clypeasteroids, Telford (1990) showed that the probability of a foraging podium contacting a particle of any particular size class depends on the contribution that class makes to the planar (projected) area of the sediment, not to its volume or mass contribution. In addition, our data consist of frequency distributions, not masses. Conventional deposit feeding data consisting of the continuous variable, mass, are best treated by analysis of variance. Frequency data are best analyzed by the *G*-statistic or log-likelihood ratio test.

According to our analysis, all gut samples differ significantly from the native sediment and from each other (Table II). Thus we have one requirement to establish selectivity, that is, statistical significance. But there is no consistent pattern of preferred size classes and no correlation of ingested particle sizes with test dimensions. Although all of the individual and the pooled gut contents exhibit a statistically significant difference from the sediment, the sheer magnitude of the *G* values (Table III) indicates that particle collection probably occurs in proportion to their areal representation rather than to volume or simple frequency. Making this assumption, we examined the distribution of *G* values by stochastic modeling: we analyzed 10,000 randomly drawn samples to compare with the observed pooled gut contents. Our stochastic model suggests that randomly drawn particle arrays will usually appear to be significantly different to the parent sediment and that caution is therefore needed in the interpretation of these *G*-tests. Lacking persuasive evidence to the contrary, we conclude that *C. caribaeorum* is probably a non-selective feeder: regardless of test size, all individuals appear to draw particles randomly from the sediment.

Evolution of podial particle picking

Elucidation of the feeding mechanism of a cassiduloid helps to fill in some of the gaps in our knowledge of the evolution of the PPPM described by Telford *et al.* (1985) and Telford and Mooi (1986). The major events in the history of this mechanism are depicted in Figure 6. Both the "regular" echinoids (a paraphyletic assemblage) and the earliest of the Irregularia, the holactypoids, had a well-developed, upright Aristotle's lantern that could be protruded to rasp at food materials positioned directly below the mouth. The accessory podia of regular echinoids are primarily food-holding, sensory, or locomotory structures. In some taxa, adoral concentrations of podia adjacent to the peristome help the echinoid maintain its position on hard bottoms exposed to waves and currents. These podia do not act as food-gathering organs. Although holactypoids seem to have inhabited coarse sands, the PPPM probably did not play a major role in food-gathering in these forms either. After a care-

ful analysis of the podial pore arrangements and overall morphology of *Holactypus*, Smith (1984: p. 106) states that it "fed using only its lantern to gather food, scooping up bottom sediment fairly unselectively. . ." The phylogenetic position of *Holactypus* (Smith, 1984) suggests that this behavior represents the plesiomorphic condition for the holactypoids. However, the extant, derived holactypoid *Echinoneus* has secondarily lost the lantern. According to Rose (1978: p. 302), sediment particles were "picked up by the nearest tube-feet and passed along the ambulacra to the mouth." Therefore, the PPPM apparently plays a major role in the feeding mechanism of *Echinoneus*. This represents a striking convergence with other lanternless Irregularia. In the spatangoids and holasteroids, which generally inhabit very fine sands or mud, the particle-picking role is restricted to the penicillate phyllopodia, which are capable of collecting large numbers of particles with each application of a podium to the sediment (Nichols, 1959; Smith, 1984).

Although the particle-gathering function of the phyllopodia is still very important to cassiduloids, they were among the first echinoids to employ accessory podia as particle-gathering organs. *C. caribaeorum* illustrates this capability very well in its use of accessories to both probe and pull particles towards the test and pass them from podium to podium towards the mouth. The elaboration of the PPPM from this earlier cassiduloid pattern involved increasing the number of accessory podia in contact with the sediment. The extinct oligopygoids (not shown in Fig. 6) had already multiplied the number of accessory podia in each ambulacrum through an elaborate plate compounding system (Kier, 1967), thereby increasing the role of the accessories in the PPPM. The clypeasteroids, in breaking the "one-podium-per-plate rule" (Mooi, 1990a), were able to vastly increase the number of accessory podia. Each accessory could operate as part of the PPPM by picking up one or two particles at a time. In some clypeasteroids, there may be up to 160 food-gathering podia per mm², giving some individuals hundreds of thousands of such podia on the oral surface, all acting as parts of miniature "bucket brigades" as they pass particles towards the mouth. Particularly in species of Mellitidae, accessory podia can cover 40% of the oral surface (Telford and Mooi, 1986). In these forms, accessories can be elaborated into several types, such as the long and short barrel-tipped podia typical of *Leodia* and *Encope*. Mooi (1986b) distinguished between accessory podia from the aboral surface and ambitus, and barrel-tipped podia from the oral surface, but the latter undoubtedly represent modifications of the former. In the scutelline clypeasteroids, the PPPM has become a sophisticated operation that relies exclusively on accessory podia and their derivatives to gather particles and pass them into the food grooves (Telford *et al.*, 1985).

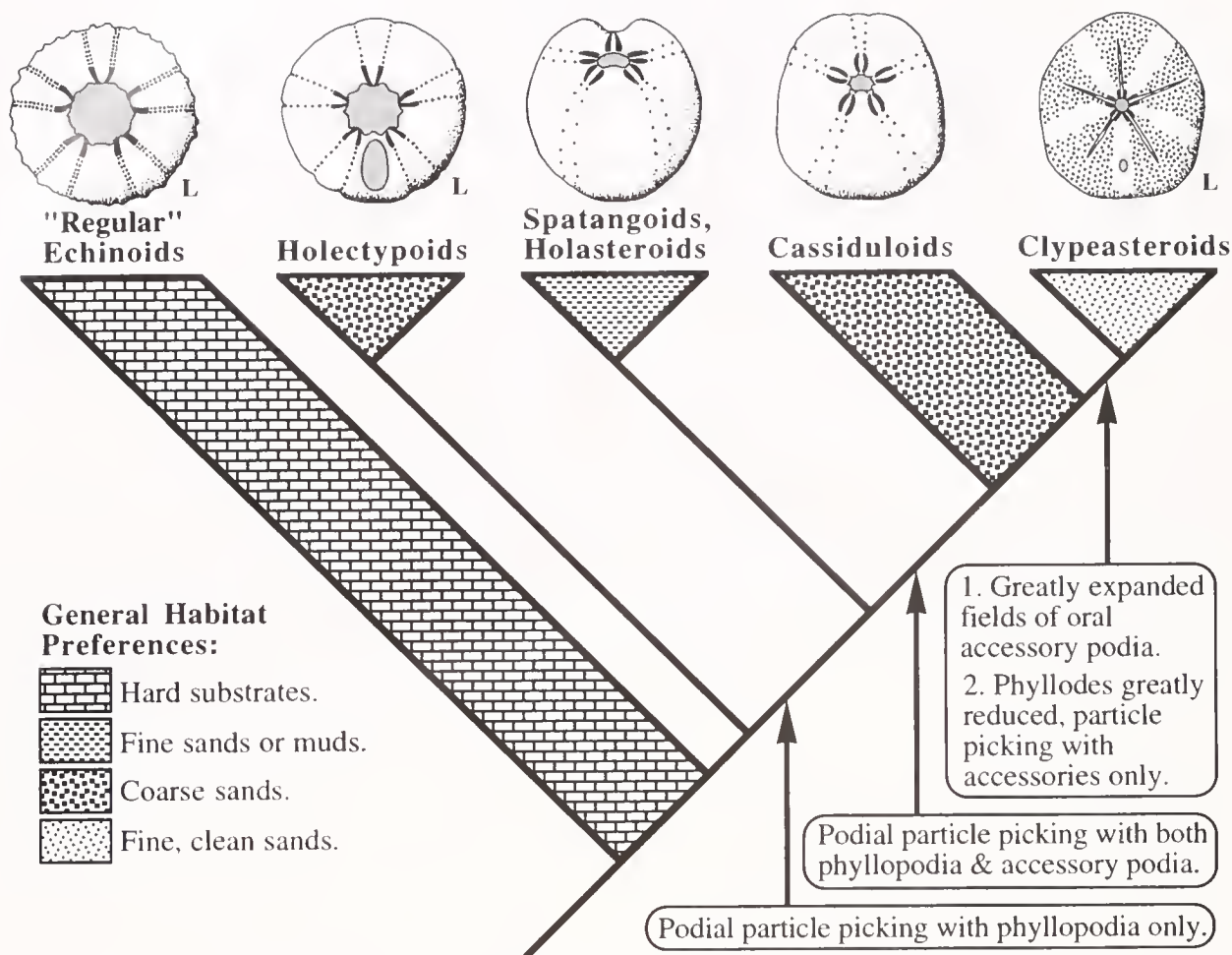


Figure 6. Phylogeny of podial particle picking in the major extant clades of Echinoidea. Arrows indicate positions of events in evolution of particle picking listed within boxes. In small figures of echinoids, mouth and anus are shaded, phyllodes are shown in solid black, accessory podial pores are indicated by dots in ambulacra, "L" indicates taxa with an Aristotle's lantern in the adult. Phylogeny after Smith (1984).

In most clypeasteroids there is a small set of slightly enlarged podia [which Mooi (1986b) called "large food groove podia"] adjacent to the peristome, but just distal to the buccal podia. These podia are similar in overall morphology and histology to other food groove podia, but are about twice their size. Large food groove podia are not involved in particle collection; rather, they help guide strings of aggregated food material into the mouth. Phelan (1977) suggested that the phyllodes of cassiduloids were homologous with the entire podial field on the oral surface of clypeasteroids. However, accessory podia are modified into phyllopodia during the ontogeny of cassiduloids, a process also responsible for the differentiation of the large food groove podia of clypeasteroids. The rest of the accessory podia in the expansive ambulacral fields of clypeasteroids do not undergo this modification. These developmental patterns, as well as the overall anatomy and arrangement of the phyllopodia in cassiduloids, indicate that their phyllodes are not homol-

ogous with the entire podial field of clypeasteroids. Because they are intermediate in size between the buccal podia and accessory podia, and because they form by differentiation of accessory podia associated with the rest of the ambulacrum, the large food groove podia of clypeasteroids are the most likely candidates for homologues of the phyllopodia of cassiduloids. The large food groove podia of clypeasteroids probably represent a greatly reduced phyllode (Fig. 6). If so, then this reduced phyllode is homologous with the phyllode of spatangoids and cassiduloids.

Acknowledgments

This research was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Operating Grant #4696 to M.T. and by California Academy of Sciences In-house Research Funds as well as NSERC Postgraduate and Postdoctoral Fellowships to R.M.

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Effect of Larval Swimming Duration on Success of Metamorphosis and Size of the Ancestrular Lophophore in *Bugula neritina* (Bryozoa)

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Abstract. There is a growing realization that events during one portion of an organism's life cycle can have both subtle and dramatic effects on other stages in the life history. Lethal and sublethal effects associated with the duration of larval swimming in marine invertebrates were examined for the bryozoan *Bugula neritina*. Larvae were kept swimming up to a maximum of 28 h at 20°C by exposure to continuous bright fluorescent illumination. At 4-h intervals, samples of 20–40 larvae were removed from bright illumination and were exposed to seawater containing 10 mM excess KCl, an inducer of metamorphosis in this species. Over the first 12 h of larval swimming, an average of about 90% of the larvae initiated and completed metamorphosis; at 16 h, the percentage of larvae initiating and completing metamorphosis dropped significantly. By 28 h, about half of the larvae were initiating metamorphosis, whereas only one-fifth were completing metamorphosis. Larval swimming duration also significantly affected the duration of metamorphosis. By 30 h of larval swimming, individuals were taking about 25% longer to complete metamorphosis. Compared to ancestrulae that developed from larvae that were induced to metamorphose shortly after the onset of swimming, those that swam for greater than 8 h had significantly smaller lophophores. For example, by 28 h of larval swimming the ancestrular lophophore decreased in height, surface area, and volume by about 25%, 40% and 55%, respectively. This marked decrease in lophophore size may ultimately affect the ability of juveniles to sequester food, compete for space, and attain reproductive maturity. Thus, increasing the duration of

larval swimming affects both metamorphosis and the development of postlarval structures, which may ultimately influence colony fitness.

Introduction

Many sedentary or sessile marine invertebrates possess a larval stage that on an ecological time scale can function to (1) extend species ranges, (2) connect otherwise geographically separated adult populations, (3) alleviate parent-offspring and intraspecific competition, and (4) facilitate the recolonization of disturbed habitats. The longer a larva spends in the plankton, however, the greater the chance that it will incur a cost due to physiological stress, starvation, predation, and advection away from suitable sites (Thorson, 1950; Rumrill, 1990; Morgan, 1995). Thus, theoretically a trade-off exists between the benefits of dispersal and its attendant costs (see Vance, 1973; Strathmann, 1985, for models examining life history strategies).

Recent evidence suggests that, in addition to lethal effects, there are also sublethal costs associated with duration of the larval swimming stage, especially for species with nonfeeding larvae (reviewed by Pechenik, 1990). Sublethal costs influencing juvenile fitness have yet to be incorporated into models of life-history strategies and have not been rigorously characterized empirically. Presumably any sublethal costs associated with remaining in the plankton are due to effects of larval senescence or depletion of energy reserves to a level at which juveniles have reduced fitness.

Feeding larvae should be buffered against costs related to the depletion of energy stores, because they can re-

plenish their energy reserves continuously (Highsmith and Emlet, 1986; Pechenik and Eyster, 1989). However, they are unable to extend their lives indefinitely, and eventually they either senesce and die or undergo spontaneous metamorphosis (Pechenik, 1990). Because of the limited energy supply (barring use of dissolved organic material [DOM]), aplanktotrophic larvae, *i.e.*, larvae that do not rely on particulate food,¹ have more rigid constraints on the amount of time they can spend in the plankton.

A larva is considered to be in an extended swimming period when it is physiologically capable of responding to cues that elicit metamorphosis, but instead continues swimming in the absence of those cues. Marine invertebrates do not possess endogenous clocks that control the duration of the larval period, but instead they metamorphose in response to cues associated with a favorable adult habitat (Scheltema, 1974; Hadfield, 1978; Morse and Morse, 1984; Chia, 1989; Pawlik, 1992). Once competent to metamorphose, a larva can remain in the swimming phase for one of two reasons: (1) it has not encountered a suitable cue to trigger metamorphosis; or (2) it has encountered the cue, but additional factors prevent the normal metamorphic response. An example of the latter situation was provided by Young and Chia (1981). They demonstrated that competent larvae of *Bugula pacifica* delay metamorphosis in the presence of extracts of a dominant competitor, the compound ascidian *Diplosoma macdonaldi*. In either case, the benefits associated with remaining in the plankton seem readily apparent: a larva is able to synchronize the onset of metamorphosis with encountering a favorable site. However, the longer a larva is in the plankton the greater its exposure to the potential lethal and sublethal effects of a planktonic existence (Rumrill, 1990; Morgan, 1995).

Because planktotrophic larvae can feed on particulate matter throughout the larval stage, they should not incur substantial sublethal costs; aplanktotrophic larvae, on the other hand, have finite energy reserves, and thus should incur these costs quicker and with more severity.

¹I propose the term *aplanktotrophic* to include larvae that are either lecithotrophic, translocational, or adelphophagic. Lecithotrophic larvae acquire nutrients (yolk) during oogenesis; translocational larvae have nutrients transferred to the developing embryo after fertilization; and adelphophagic larvae feed on siblings during encapsulation or brooding. These terms refer to the specific processes by which larvae obtain their energy reserves. The inappropriate synonymy of the terms lecithotrophy and nonfeeding is commonly encountered in the literature. Clearly all nonfeeding larvae are not lecithotrophic. The term *aplanktotrophic* is a more appropriate general classification for larvae that derive their energy reserves from sources other than the plankton, and it emphasizes the underlying similarity of these larvae (*i.e.*, not feeding on planktonic particulate matter). From an energetic standpoint, aplanktotrophic larvae are independent of particulate food in the plankton.

This generalization is supported by the few studies that have addressed the issue explicitly. For example, Highsmith and Emlet (1986) found no significant correlation between delay time and juvenile growth rate in the sand dollar *Echinorachnius parma*, which has planktotrophic larvae. Also, Pechenik and Eyster (1989) found no significant differences in average survival, feeding, respiration, or growth rates between juveniles of the planktotrophic gastropod *Crepidula fornicata* that were induced to metamorphose shortly after attaining competence and those that were kept swimming until metamorphosis occurred spontaneously. Results of these two studies suggest that planktotrophic larvae can have an extended larval swimming period without incurring substantial cost to juvenile fitness.

In contrast, species with aplanktotrophic larvae can incur sublethal costs as the duration of the larval swimming period increases. For example, in 12 out of 14 cases Woollacott *et al.* (1989) demonstrated that after 10 h of swimming, larvae of *B. stolonifera* developed into juveniles that grew significantly slower than juveniles developed from larvae that had a swimming period of 6 h. For the barnacle *Balanus amphitrite*, prolonging the swimming period of cyprids for 3–5 days dramatically depressed juvenile growth rate compared to controls (Pechenik *et al.*, 1993). Conversely, Pechenik and Cerulli (1991) found that prolonging the swimming period of the aplanktotrophic larvae of the marine polychaete *Capitella* sp. I for up to 216 h had no effect on postmetamorphic growth rate, time to first reproductive activity, or fecundity. However, increasing larval swimming time did significantly decrease postsettlement survivorship from 100% (not prolonged) to 12.5% (prolonged for 216 h). Although those individuals that survived incurred no sublethal costs, there was a substantial postlarval mortality.

Clearly, being able to initiate and to complete metamorphosis is central to survival. More subtle, however, are the sublethal effects associated with a prolonged period of larval swimming. Intra- and interspecific competition between sessile invertebrates for space and food is directly related to size (Buss, 1979; Buss and Jackson, 1981). In addition, for *Bugula neritina*, reproductive maturity is attained only after a minimum number of bifurcations (Keough, 1987). Therefore, an individual's fitness may be compromised by being smaller after metamorphosis or by taking longer to metamorphose.

The effects of the duration of larval swimming were assessed for the cheilostome bryozoan *B. neritina*, which has an aplanktotrophic larva that acquires its nutrient reserves translocationally through a placenta-like system (Woollacott and Zimmer, 1975). Specifically, I examined (1) duration of metamorphosis; (2) ability of larvae to initiate metamorphosis; (3) ability of individuals to com-

plete metamorphosis; and (4) size of and tentacles in the ancestrular lophophore. Because larvae of *B. neritina* are aplanktotrophic and therefore limited energetically, I predicted (1) a positive correlation between larval swimming duration and time required to complete metamorphosis (a result of the utilization of less labile energy sources to complete metamorphosis); (2) an inverse correlation between larval swimming duration and ability to initiate and complete metamorphosis; and (3) a negative correlation between size of the lophophore and duration of the larval swimming stage. These factors influence the ability to sequester food, compete for space, and attain reproductive maturity, which undoubtedly affects an individual's fitness.

Materials and Methods

Gravid colonies of *Bugula neritina* were collected from the undersides of floating docks near the Smithsonian Marine Station in Fort Pierce, Florida, from February through April 1995 and in February 1996. Colonies were shipped to Cambridge, Massachusetts, and were maintained in the laboratory in plastic aquaria at 20°C in darkness with continuous aeration. No food was provided for the colonies.

Larvae were obtained from pools of colonies to foster genetically heterogeneous populations for the experiments. Colonies were removed from the dark, placed in glass bowls with 1.5 l of seawater, and exposed to fluorescent light. Within 30 min of illumination, larvae appeared; by 2 h, release was complete. *B. neritina* larvae are positively phototactic on release, and this behavior facilitated their collection because they aggregated on the illuminated side of dishes. Larvae were collected and dispensed to experimental vessels with pipettes. Experiments were started within 1.5 h after the appearance of larvae; therefore, at most, larvae would differ in age by 90 min. Larvae used in experiments were obtained only from parent colonies kept in the laboratory less than 6 days, and parent colonies were re-used for releases over this 5-day period in the laboratory.

Larvae of *B. neritina* did not initiate metamorphosis when exposed to bright fluorescent illumination; thus by removing larvae from the illumination at regular intervals, it was possible to create populations that differed in the length of swimming time. Following release, larvae were transferred to a glass finger bowl containing 250 ml of 0.2- μm -filtered seawater. The finger bowl was illuminated from below using three 50-watt fluorescent Vita Lites. Illumination levels ranged from 130 to 145 $\mu\text{E m}^{-2}\text{s}^{-1}$. Due to their small size, the larvae in each condition could only be counted accurately after the experiment was over. Approximately 20–40 larvae were removed from the bright fluorescent illumination at 4-h

intervals for 28 h. They were then pipetted into glass Stendor dishes containing 20 ml of 0.2- μm -filtered seawater with an excess of 10 mM KCl, an inducer of bryozoan metamorphosis (Wendt and Woollacott, 1995). The swimming times reported are minimum estimates in that larvae did not initiate metamorphosis instantaneously. However, preliminary experiments showed that > 70% of the larvae initiated metamorphosis within the first hour after transfer. Initiation and completion of metamorphosis were determined for each sample condition after 72 h (see below on how initiation and completion are defined).

Each time condition was sampled and metamorphosed individuals were "relaxed" in 7.5% MgCl_2 . Tentacle length, lophophore crown diameter, and lophophore base diameter were measured for each ancestrula (Fig. 1). These parameters were used to calculate height, volume, and surface area of the lophophore on the basis of the following equations (Beyer, 1987),

$$\text{Height} = \sqrt{c^2 - (1/4)(a - b)^2} \quad (1)$$

$$\text{Volume} = \pi h[a^2 + ab + b^2]/12 \quad (2)$$

$$\text{Surface area} = \pi c(a + b)/2 \quad (3)$$

where a is diameter of the lophophore at its crown, b is

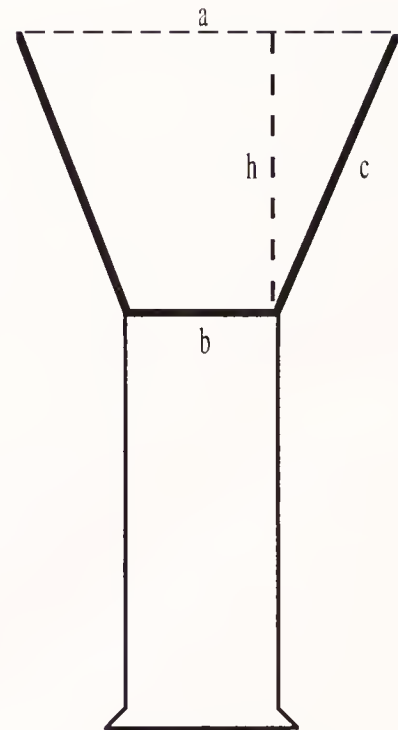


Figure 1. Schematic of an ancestrula of *Bugula neritina* showing the measured parameters, crown diameter (a), base diameter (b), and tentacle length (c), and the calculated parameter, height (h). The height was calculated using equation 1 in Materials and Methods.

diameter of the lophophore at its base, c is tentacle length, and h is height of the lophophore.

An additional three trials of this experiment (using 270 individuals) were carried out to extend the larval swimming period by 12 more hours and to increase the number of individuals at later time points. This experiment followed an identical protocol, but larvae were sampled only at 0 h, 30 h, and 40 h. Due to the difference in sampling times, data from these three trials are presented separately.

A second experiment was conducted to determine if the time to metamorphosis changed as a function of larval swimming duration. In this experiment larvae were sampled at 10-h intervals, and they were transferred to and kept separate in 2-ml multiwelled dishes. Individual larvae were followed on an hourly basis to provide an accurate determination of when metamorphosis was initiated and completed.

Attachment (often referred to as settlement in the literature) of bryozoan larvae is tightly coupled to metamorphosis in that attachment is not reversible and is marked by eversion of the metasomal (internal) sac. This is the first morphogenetic movement of metamorphosis in larvae of *Bugula* spp. and other bryozoans (Zimmer and Woollacott 1977). Thus, attachment in bryozoans is not exclusively a behavioral change associated with substratum exploration. I will hereafter refer to this process as the initiation of metamorphosis. Metamorphosis was considered complete after the ancestrula everted its lophophore.

The number of larvae that initiated and completed metamorphosis in each treatment was expressed as a percentage. The results of each of the replicates were plotted individually to determine between-trial similarity. Without exception, the general trends were similar in each of the replicates, and the replicates were thus treated as single data sets for statistical analysis and graphing. Data from within treatments did not differ significantly from a normal distribution, so a factorial ANOVA was performed to identify heterogeneity of variances within the data set. Fisher's protected least significant difference (PLSD), a *post hoc* test, was used to identify sources of such variation among the data.

Results

Duration of metamorphosis

Time required from initiation to completion of metamorphosis increased significantly for each 10-h increase in larval swimming period (ANOVA, $F = 55.7$; $P < 0.0001$; Fisher PLSD, 0 vs. 10, $P = 0.004$, 10 vs. 20 and 20 vs. 30, $P < 0.0001$). Mean duration of metamorphosis was 39 ± 0.4 h, 42 ± 0.3 h, 46 ± 0.7 h, and 49 ± 0.9 h for individuals that developed from larvae swimming for

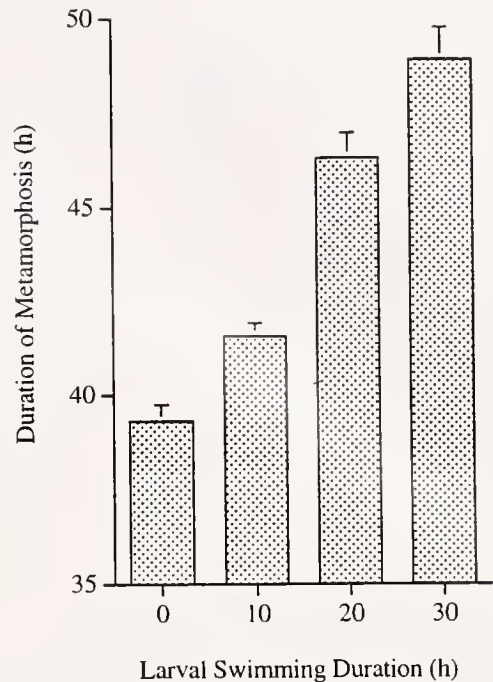


Figure 2. *Bugula neritina*. Duration of metamorphosis versus larval swimming period. Individual larvae were followed on an hourly basis from initiation (attachment by eversion of the metasomal sac) to completion (eversion of the lophophore) of metamorphosis. All time classes are significantly different from each other (bars = 1 standard error; $n = 26$ –35 for each condition).

0, 10, 20, and 30 h, respectively (Fig. 2). Cumulative percent completion of metamorphosis was sigmoidal, although the curve was right-shifted for each successive larval swimming period (Fig. 3). The amount of time for 50% of the individuals to complete metamorphosis (K_0) was approximately 37.5, 40.5, 44.0, and 49.0 h for individuals that developed from larvae swimming 0, 10, 20, and 30 h, respectively (Fig. 3). Duration of metamorphosis ranged from 36 to 62 h.

Initiation and completion of metamorphosis

An increased larval swimming period significantly affected the percentage of individuals initiating and completing metamorphosis. ANOVA revealed significant heterogeneity of variances for percent initiation and percent completion of metamorphosis over the duration of the experiment (Table 1). On average, close to 95% of the larvae initiated metamorphosis through 12 h (Fig. 4). At 16 h, $67 \pm 7\%$ (mean $\pm$ standard error) of the larvae were initiating metamorphosis ($P = 0.01$; Fisher PLSD). After 16 h the percentage of larvae initiating metamorphosis decreased slightly, although the variability between trials was such that no significant decline occurred between 16 and 28 h. On average for 0 through 12 h, the percentage

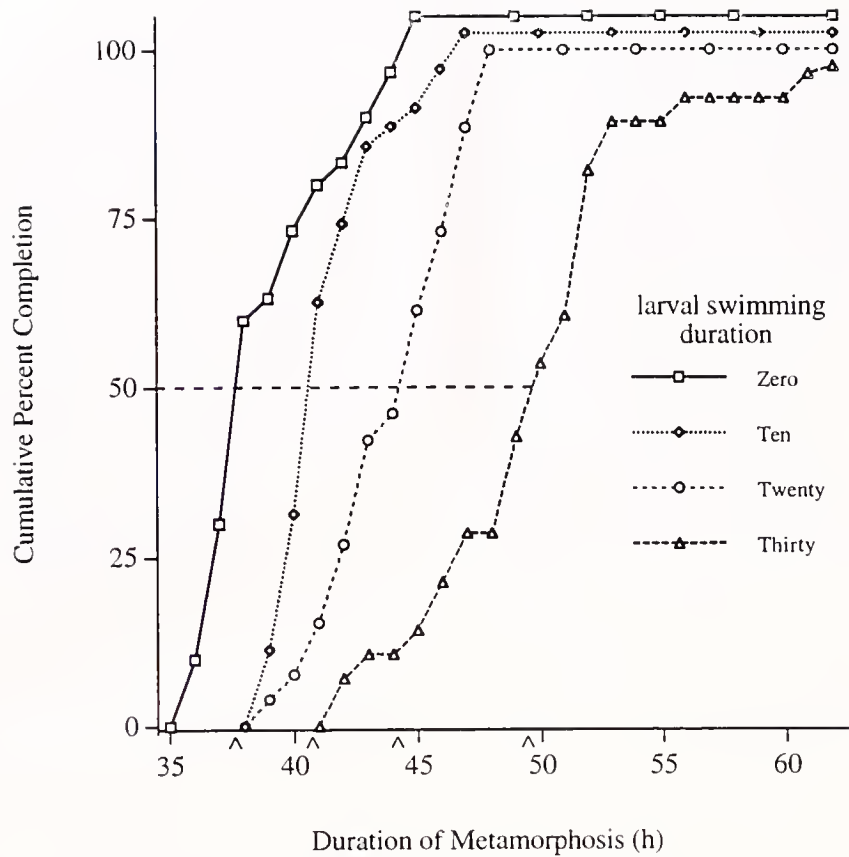


Figure 3. *Bugula neritina*. Cumulative percent completion of metamorphosis versus larval swimming period. K_c values, the time for 50% of the individuals to complete metamorphosis, were approximated by connecting 50% completion to the x-axis. Lines at 100% completion are staggered for clarity. $n = 26\text{--}35$ individuals for each swimming time.

of individuals completing metamorphosis was almost identical to the number of larvae initiating metamorphosis (Fig. 4). The percentage of individuals completing metamorphosis declined significantly at 16 h to $52\% \pm$

12% (mean $\pm$ standard error; $P = 0.0004$; Fisher PLSD). In contrast to initiation, however, mean percentage of individuals completing metamorphosis declined significantly from 16 h to 24 and 28 h (mean $\pm$ standard error, 52 ± 12 , 22 ± 7 , and 17 ± 3 , respectively; $P = <0.003$; Fisher PLSD).

Table I

One-factor ANOVA results for the effect of larval swimming duration on duration of metamorphosis, on initiation and completion of metamorphosis, on the height, surface area, and volume of the lophophore, and on the measured parameters of the lophophore

Parameter	df	F-test	P-value
Duration of metamorphosis	3	55.75	<0.0001
Initiation of metamorphosis	7	5.97	0.0002
Completion of metamorphosis	7	25.63	<0.0001
Tentacle length	7	9.98	<0.0001
Lophophore diameter (top)	7	9.981	<0.0001
Lophophore diameter (base)	7	4.180	<0.0003
Lophophore height	7	9.195	<0.0001
Lophophore surface area	7	10.69	<0.0001
Lophophore volume	7	10.58	<0.0001

Lophophore parameters

An increased larval swimming period significantly affected the size of the ancestrular lophophore in *Bugula neritina*. Mean tentacle length and base and crown diameters of the lophophore of the ancestrulae decreased by 25%, 24%, and 12.5%, respectively, in individuals that developed from larvae that were swimming for 28 h compared to individuals that developed from larvae induced to metamorphose shortly after the onset of larval swimming (Table II). Mean height, surface area, and volume of the lophophore decreased as a function of increased larval swimming duration (Fig. 5a, b, c). ANOVA revealed significant heterogeneity of variances for

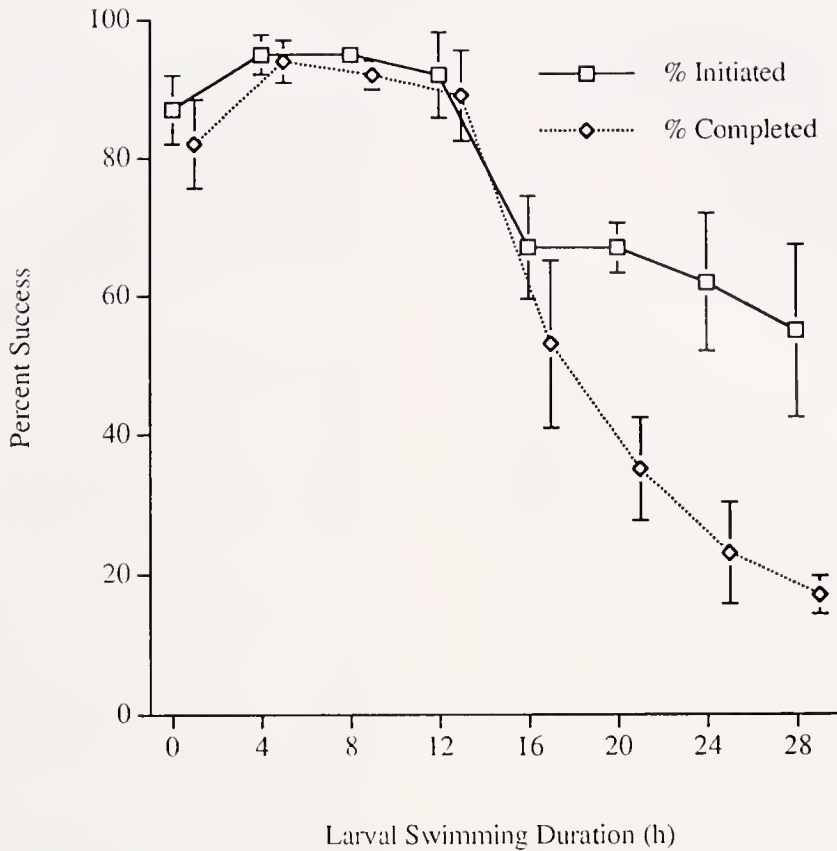


Figure 4. Percent initiation and completion of metamorphosis *versus* larval swimming duration in *Bugula neritina*. Larvae kept swimming for 16 h were less likely to both initiate and complete metamorphosis. The lines are staggered slightly for clarity (bars = 1 standard error; $n = 5$).

each of these calculated parameters (Table I), and significant declines occurred after 8 h of larval swimming. By 28 h of larval swimming the percent decrease in mean lophophore height, surface area, and volume was 25%, 40%, and 55%, respectively, compared to individuals that developed from larvae induced to metamorphose shortly after the onset of larval swimming. Maximum number of tentacles (21; range 19–21) occurred in indi-

viduals that developed from larvae induced to metamorphose shortly after the onset of larval swimming (Table II). Minimum number of tentacles (16; range 16–20) occurred in ancestrulae that developed from larvae kept swimming for 24 h prior to the onset of metamorphosis (Table II).

Significant declines were observed for all parameters in the second experiment also. After 40 h of larval swim-

Table II

Tentacle length, top and base diameter of the lophophore, and ranges of tentacle number as a function of larval swimming duration (mean $\pm$ standard error of the mean); measurements were made on ancestrulae less than 24 h post metamorphosis

Swimming Duration (h)	N	Tentacle Length (μm)	Top Diameter (μm)	Base Diameter (μm)	Tentacle # (Range:Mode)
0	28	520 $\pm$ 10	556 $\pm$ 10	152 $\pm$ 3	19–21:20
4	31	493 $\pm$ 11	535 $\pm$ 11	153 $\pm$ 2	17–21:19
8	28	467 $\pm$ 13	513 $\pm$ 11	145 $\pm$ 3	18–21:19
12	32	469 $\pm$ 12	518 $\pm$ 9	142 $\pm$ 2	17–21:20
16	23	442 $\pm$ 12	499 $\pm$ 13	145 $\pm$ 3	17–21:19
20	23	412 $\pm$ 16	474 $\pm$ 20	139 $\pm$ 4	17–21:19
24	15	400 $\pm$ 16	424 $\pm$ 15	142 $\pm$ 3	16–20:18, 19
28	8	390 $\pm$ 27	423 $\pm$ 21	133 $\pm$ 4	18–20:20

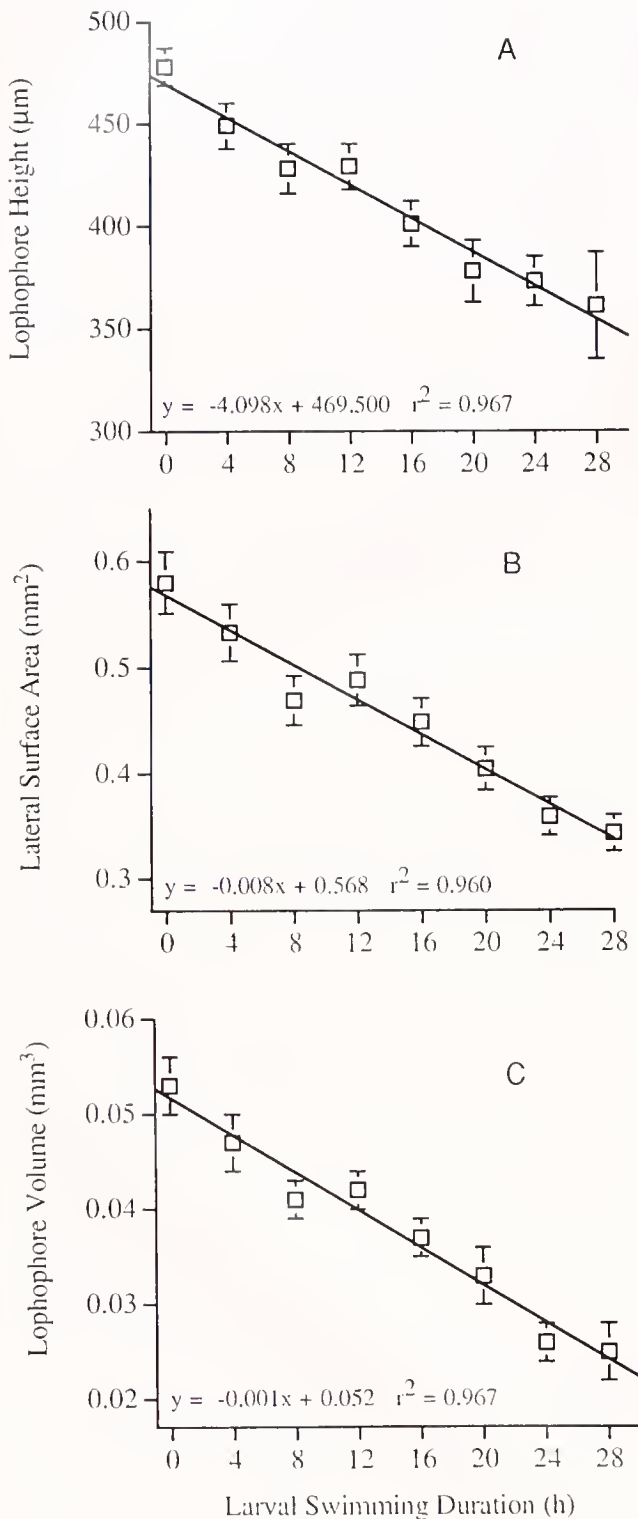


Figure 5. *Bugula neritina* Height (A), surface area (B), and volume (C) of lophophore as a function of larval swimming duration in *Bugula neritina*. Samples of larvae were taken every 4 h, at which time they were induced to metamorphose by exposure to 10 mM excess KCl in seawater. Each datum is the mean of 8–32 individuals pooled from 5 replicates (bars = 1 standard error).

ming, the mean height, surface area, and volume decreased by *ca.* 30%, 50%, and 62%, respectively, when compared to those of individuals that developed from larvae induced to metamorphose shortly after the onset of larval swimming (ANOVA; $F = >100.00$, $P = <0.0001$ for each parameter; $n = 3$; a total of 107, 143, and 20 individuals were measured for 0, 30, and 40 h, respectively).

Discussion

Duration of metamorphosis

Duration of metamorphosis on average increased as a function of larval swimming period (Figs. 2 and 3). After 30 h of larval swimming, individuals were taking almost 10 h longer to complete metamorphosis than those induced shortly after the onset of larval swimming. The observed increase in duration of metamorphosis is probably due to the utilization of less labile energy sources to complete metamorphosis. In *Bugula* spp. certain larval tissues (*e.g.*, the corona, ciliated epithelia, and vesicular collarettes) are transitory. At the onset of metamorphosis these tissues are internalized and ultimately undergo histolysis (Woollacott and Zimmer, 1978). Individuals that swim longer before initiating metamorphosis deplete labile energy stores to a greater degree and, therefore, they must rely more heavily on these transitory tissues for the energy necessary to complete metamorphosis. It is likely that the biochemical processes needed to histolyze these tissues increase the duration of metamorphosis. Whether the increase in duration of metamorphosis compromises juvenile fitness has yet to be determined.

Initiation and completion of metamorphosis: lethal effects and the mechanisms governing abilities to initiate and complete metamorphosis

These data document a substantial lethal cost to *B. neritina* in that a significant portion of larvae lose the ability over time to initiate or complete metamorphosis. Woollacott *et al.* (1989) found similar lethal costs in *B. stolonifera* in that 40% of larvae lost metamorphic competence after 10 h of swimming. A loss of competence was also observed after 24 h of larval swimming in *Celleporella hyalina*, another cheilostome bryozoan (Orellana and Cancino, 1991). Pechenik and Cerulli (1991) found that delaying metamorphosis of the aplanktotrophic larvae of the marine polychaete *Capitella* sp. caused an 87% decrease in postsettlement survivorship in individuals that developed from larvae that were swimming for 216 h, compared to individuals that developed from larvae induced to metamorphose shortly after becoming competent.

The mechanisms governing the ability of larvae to ini-

tiate metamorphosis and the ability of larvae to complete metamorphosis are unknown. Two hypotheses have been presented to explain loss of metamorphic competence: (1) an energetic hypothesis stating that larvae lose competence as a result of depleting energy reserves during swimming (Lucas *et al.*, 1979; Pechenik, 1990; Jaeckle, 1994; Pawlik and Mense, 1994); and (2) a sensory hypothesis stating that larvae are unable to respond to cues due to degradation of the receptor or other limiting elements in the transduction pathway (Pechenik, 1980; Pechenik and Fried, 1995). These processes are probably not mutually exclusive given that the ability of larvae to maintain a functional receptor apparatus is also presumably dependent on energy.

The ability of *B. neritina* to successfully initiate metamorphosis may not depend directly on depletion of energetic reserves. If ability to initiate metamorphosis were under energetic constraints, then as larvae continue to swim, one might expect a larger portion to deplete their reserves below the level needed for initiating metamorphosis. One should then observe a continuous decrease in successful initiation throughout the duration of larval swimming. Such a pattern, however, was not observed. Specifically, after 16 h of larval swimming, the ability to initiate metamorphosis was not negatively correlated with larval swimming duration (Fig. 4). Further data for addressing energetic constraints as an explanation for loss of metamorphic competence (*i.e.*, ability to initiate) in *B. neritina* is provided by Jaeckle (1994) in his respirometry work with larvae. If we assume an average respiration rate of $0.118 \text{ mJ larva}^{-1} \text{ h}^{-1}$ (calculated from data in table 5; Jaeckle, 1994) and an average energy content of $15.24 \text{ mJ larva}^{-1}$ for larvae of *B. neritina* (Jaeckle, 1994), by 16 h of swimming, larvae will have depleted 12% of their energy content. Whether a 12% decrease in larval energy reserves is sufficient to cause a loss of metamorphic competence is unknown, but on average over 60% of larvae retained metamorphic competence at this time. These data suggest that an alternative mechanism (*e.g.*, receptor degeneration) may be governing the loss of metamorphic competence in *B. neritina*.

Although the ability to initiate metamorphosis may not be under energetic control, the ability to complete metamorphosis is likely to be constrained energetically. The fact that completion of metamorphosis continues to decrease significantly throughout the duration of larval swimming indicates that energetic limitations exist. The exact portion of individuals that initiate but fail to complete metamorphosis is represented by the divergence of the two curves after 16 h (Fig. 4). On average by 28 h of larval swimming, only 30% of the individuals that initiated metamorphosis successfully completed it. The divergence between the ability of larvae to initiate and complete metamorphosis after 12 h demonstrates that

although larvae can successfully respond to cues and initiate metamorphosis, they are unable to complete it (Fig. 4). This observation suggests that factors controlling the ability to initiate metamorphosis are distinct from those controlling the ability to complete it. Possibly, as the population of larvae continue swimming, a larger portion deplete their energy reserves beyond the amount needed to complete metamorphosis. This energetic dependency is further supported by the observation that larvae die at various stages during metamorphosis.

Thus, for aplanktotrophic bryozoan larvae, ability to initiate metamorphosis appears independent of energy reserves and may depend on degradation of some key element in the receptor pathway responsible for site recognition. In contrast, ability to complete metamorphosis may depend on larval energy reserves and, therefore, indirectly on the duration of the larval swimming period.

In light of recent studies on the uptake of dissolved organic matter (DOM) in invertebrate larvae (Langdon, 1983; Manahan, 1983, 1989, 1990; Jaeckle and Manahan, 1989a, b; Shilling and Manahan, 1990; Welborn and Manahan, 1990; Ronnestad *et al.* 1992; Fenaux *et al.*, 1994; Hoegh-Guldberg, 1994; Jaeckle, 1994, 1995b), discussions of larval energetics must incorporate the potential metabolic contributions of this alternative energy source. It should be emphasized, however, that although the transport of DOM by larvae is well established, the metabolic use of this additional energy pool and its relevance to larval ecology remain equivocal (Pechenik, 1990; Jaeckle, 1995a). Experiments are needed that compare metamorphic competence, maximum swimming duration, and size after metamorphosis between individuals that have access to DOM and those that do not.

Lophophore parameters: sublethal effects and their potential costs to juvenile fitness

In addition to the lethal costs, such as the loss of metamorphic competence and the inability of larvae to complete metamorphosis, there exist more subtle, sublethal costs to juvenile fitness. These results document that mean size of the ancestrular lophophore decreases as larval swimming period increases (Table II, Figs. 5a, b, c). After a larval swimming period of 28 h, there were significant reductions in all parameters of the lophophore. Sublethal costs to juvenile fitness have also been demonstrated for the congener *B. stolonifera* (Woollacott *et al.*, 1989) and the barnacle *Balanus amphitrite* (Pechenik *et al.*, 1993). The observed reduction in lophophore size may affect the ability of ancestrulae to clear food particles from their surrounding medium. The surface area and volume of the lophophore have not been examined explicitly as variables in bryozoan feeding rates, although

the height of the lophophore has been shown to be positively correlated with particle velocity (Best and Thorpe, 1986). The observed decrease in height (Fig. 5a) may have a marked effect on competitive ability of ancestrulae, because larvae commonly recruit in a clumped fashion (Keough, 1984; Roberts *et al.*, 1991). Furthermore, Keough (1987) found that postmetamorphic mortality of *Bugula neritina* is as high as 70% within the first week. He attributed this high mortality rate to microhabitat differences, but further evaluation in the context of post-metamorphic conspecific competition is needed. Also, because reproduction in *B. neritina* occurs only after a colony reaches a certain minimum size (*ca.* 7 bifurcations) and total reproductive output in colonial marine organisms is a function of overall colony size (Keough, 1989), individuals with a greater ability to gather food will attain the reproductive threshold sooner. My results demonstrate that increasing the duration of larval swimming in the aplanktotrophic larvae of *B. neritina* causes a marked decrease in lophophore size, which may ultimately compromise colony fitness.

This study clearly demonstrates the effects of extended larval swimming on the metamorphosis and development of postlarval structures in marine invertebrates. The lethal and sublethal effects accrued to individuals as a result of events that occur during the larval period have also been shown in fish (McCormick and Molony, 1992) and amphibians (Audo *et al.*, 1995). Understanding the scope of these effects in marine invertebrates as well as fish and amphibians will require studies of the physiological underpinnings of these effects and the costs to juvenile fitness.

Acknowledgments

I thank Sherry Reed at the Smithsonian Marine Station at Link Port, Fort Pierce, Florida, for generously collecting all the animals used in these experiments. Colleen Cavanaugh, Edward Selig, Michael Temkin, and Robert Woollacott (all of Harvard University) provided thoughtful discussions and comments. Jan Pechenik (Tufts University) and an anonymous reviewer offered valuable comments and criticisms that greatly improved this paper.

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Two Kinds of Active Factor in Crab Hatch Water: Ovigerous-Hair Stripping Substance (OHSS) and a Proteinase

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Abstract. The embryos of intertidal and estuarine crabs are clustered on the ovigerous seta of the female, where they are ventilated for 2–4 weeks by the female's abdomen. When the embryonic development is complete, hatching occurs and zoea larvae are released into the water. This study indicates that the crab hatch water (*i.e.*, the filtered medium into which zoeas were released) contains at least two kinds of active substance: OHSS (ovigerous-hair stripping substance) and a proteolytic enzyme. Both factors were separated by gel filtration. Powdered fragments of egg capsule were digested by proteinase, suggesting that this enzyme actually acts on the egg capsule. But this activity was at a very low level compared with casein digestion. The proteinase might be digesting the thin, sticky layer enclosing the embryo and would not act on the thick, tough layer constituting the main component of the egg capsule. Therefore, a proteolysis of such low activity could not be expected to cause the egg capsule to rupture.

Introduction

Embryos of intertidal and estuarine crabs are clustered on the ovigerous setae of the female and incubated there for 2–4 weeks. When the embryonic development is complete, hatching occurs and zoeas are liberated into the water by the special fanning behavior of the female (Saigusa, 1982). The timing of hatching is controlled by the circatidal clocks of both the female and the embryos (Saigusa, 1992a, b, 1993), but the physiological mecha-

nism underlying the timing of hatching is not known. To settle this problem, the process of hatching should be studied.

A number of investigations have indicated that the embryos secrete a proteinase upon hatching (for reviews, see Davis, 1981; Yamagami, 1988). These proteinases are thought to dissolve a portion of the egg capsule, thus rupturing it. Some of these proteinases have been purified and characterized: *e.g.*, in the sea urchin blastula (Barrett and Edwards, 1976; Lepage and Gache, 1989; Roe and Lennarz, 1990), fishes (Yamagami, 1972; Hagenmeier, 1974; Yasumasu *et al.*, 1989a, b), and amphibians (Carroll and Hedrick, 1974; Katagiri, 1975).

On the other hand, little is known about the hatching mechanism in Crustacea. Hatching of crustaceans usually occurs upon the rupture of the egg capsule. Many investigations have suggested that this is due to an increase of internal pressure caused by osmotic effects (Yonge, 1937, 1946; Burkenroad, 1947; Marshall and Orr, 1954; Davis, 1959, 1964; Anderson and Rossiter, 1969), or to the swelling of the embryos (Saigusa, 1992b).

Recently, De Vries and Forward (1991) reported that the embryos of a few estuarine crabs release a proteinase upon hatching. But in other experiments, the egg capsules after hatching showed no sign of dissolution (Saigusa, 1992b). So the role of such a proteinase in the hatching of decapod crustaceans remains obscure.

A further question is related to another active factor found in crab hatch water. Hatch water (*i.e.*, the filtered medium into which zoeas have been released) contains an active substance that causes each ovigerous hair to slip out of the investment coat that binds it to the embryo through the funiculus. The embryos is thus lost without

hatching (for details, see Saigusa, 1994, 1995). A question is whether this factor, which I call OHSS (ovigerous-hair stripping substance), is the proteinase.

I also report here that crab hatch water contains a protease. With gel filtration chromatography, the proteolytic activity is eluted in fractions different from those of OHSS. The protease certainly dissolves the debris of the egg capsule, but the activity is very weak. Consequently I have concluded that this protease does not dissolve the main components of the egg capsule.

Materials and Methods

Hatch water collection

Ovigerous females of *Sesarma haematocheir* that were expected to hatch within a few days were collected from the field at Kasaoka, Okayama Prefecture, and brought to the laboratory. They were dipped into 50% EtOH for a few minutes, for disinfection, and were individually put into beakers (8.5 cm in diameter, 12 cm in height) containing 30 ml of distilled water.

Hatching of estuarine crabs is highly synchronized; in *S. haematocheir*, all zoeas hatched within 5–30 min. As soon as hatching had been completed and the female had released all her zoea larvae into the medium, she was removed, and the medium was filtered through nylon mesh to remove larvae. This filtered medium (*i.e.*, hatch water) was accumulated in the plastic bottles and centrifuged at 15,000 rpm for 30 min to remove solid materials. The hatch water was then lyophilized, and the powder was suspended in 10 mM Tris-HCl buffer (pH 8.5). These sample solutions (concentrated hatch water) were centrifuged at 15,000 rpm for 60 min, and were stored at -20°C until used.

Gel filtration chromatography

The sample solutions were applied to a column of Sephacryl S-200 (1.3×45 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 8.5). The flow rate was 5 ml per hour, and elution of the proteins was calibrated with blue dextran and NaCl. The proteins contained in each fraction was measured at 280 nm. These experiments were all carried out at 4°C .

Casein assay

Proteolytic activity was assayed with casein (Zwilling and Neurath, 1981). The substrate was 1% casein (Ishizu Seiyaku Co., Japan) that was suspended in 100 mM Tris-HCl buffer (pH 8.5) and heated for 10–15 min in a boiling water bath. The assay mixture contained 0.2 ml of 100 mM Tris-HCl buffer (pH 8.5), 0.4 ml of 1% casein

solution, and 0.2 ml of the enzyme solution. This mixture was incubated for 30 min at 30°C , and was precipitated through the addition of 1.2 ml of 5% TCA (trichloroacetic acid). In control experiments, TCA was added to the 1% casein solution before the enzyme solution. The incubation mixture was then centrifuged at 10,000 rpm for 20 min, and the absorbance of the deproteinized supernatant was measured at 280 nm.

Assay with debris of the isolated egg capsule

Clusters of premature embryos (identified by their brown color; *e.g.*, see Saigusa, 1993, 1994) were detached from the females and folded into a sheet of nylon mesh. They were crushed and washed repeatedly with tap water to remove any embryonic tissue and yolk remaining inside of the egg capsule. The coarse fragments of the egg capsule remaining on the mesh were suspended in distilled water for one night at 4°C . Broken ovigerous setae at the bottom of the glass beaker were removed. After lyophilization, the dried samples were further crushed in a mortar and stored at -20°C .

The powder of the egg capsule debris (30 mg) was suspended in 20 ml of 100 mM Tris-HCl buffer (pH 8.5), and this suspension was used as a substrate in the assay of proteolytic activity. The assay mixture contained 0.1 ml of Tris-HCl buffer, 0.2 ml of the substrate, and 0.1 ml of the enzyme solution. This mixture was incubated for 1.5 h at 30°C and precipitated by the addition of 5% TCA (0.6 ml). In the control experiment, 5% TCA was added to the substrate before the enzyme solution. These mixtures were centrifuged at 10,000 rpm for 30 min, and the supernatant was measured at 270 nm.

Assay of OHSS

The activity of OHSS was assayed on clusters of embryos detached from ovigerous females. The ovigerous seta with their premature embryos were separated and cut into 4–6 pieces. Each cluster of embryos was placed in a well of a plastic culture dish and incubated with 0.4 ml of the solution that had eluted upon gel filtration chromatography. After 2 h of incubation at about 25°C , the embryos were transferred to a glass dish with a small quantity of distilled water, then gently pulled with a fine forceps under the stereomicroscope.

The ratio of the number of ovigerous hairs that slipped from the investment coat without breaking, to the total number of hairs on the seta was estimated for each cluster of embryos. The activity of OHSS was determined with respect to the 45% response level (ED_{50}): *i.e.*, the value at which the dose-response curve intersects the de-

fined 45% line (for further details of the assay, see Saigusa, 1995).

Results

Elution of proteolytic activity

Concentrated hatch water (1.5 ml; about five females) was fractionated on the Sephacryl S-200 column with 10 mM Tris-HCl buffer, and the proteolytic activity of each fraction was examined. As shown in Figure 1, the proteolytic activity eluted near the void volume of the column. Fractions in the latter half showed no activity.

The molecular size of this proteinase should be deter-

mined with gel filtration chromatography. But this active factor could be a product of the enzyme-substrate reaction, since hatch water is the medium into which the zoeas were hatched and released. The proteinase could be combining with substrate in the egg capsule (or on the surface of the embryos) to form conjugated proteins. So further investigations will be required to determine the molecular size of this enzyme.

Hatch water was also collected from other species (*S. pictum*, *S. dehaani*, and *Hemigrapsus sanguineus*), subjected to the same gel filtration protocol as was used with *S. haematocheir*, and assayed for proteolytic activity in the same way. Proteolytic activities were detected in the hatch water from all of the species, suggesting that the

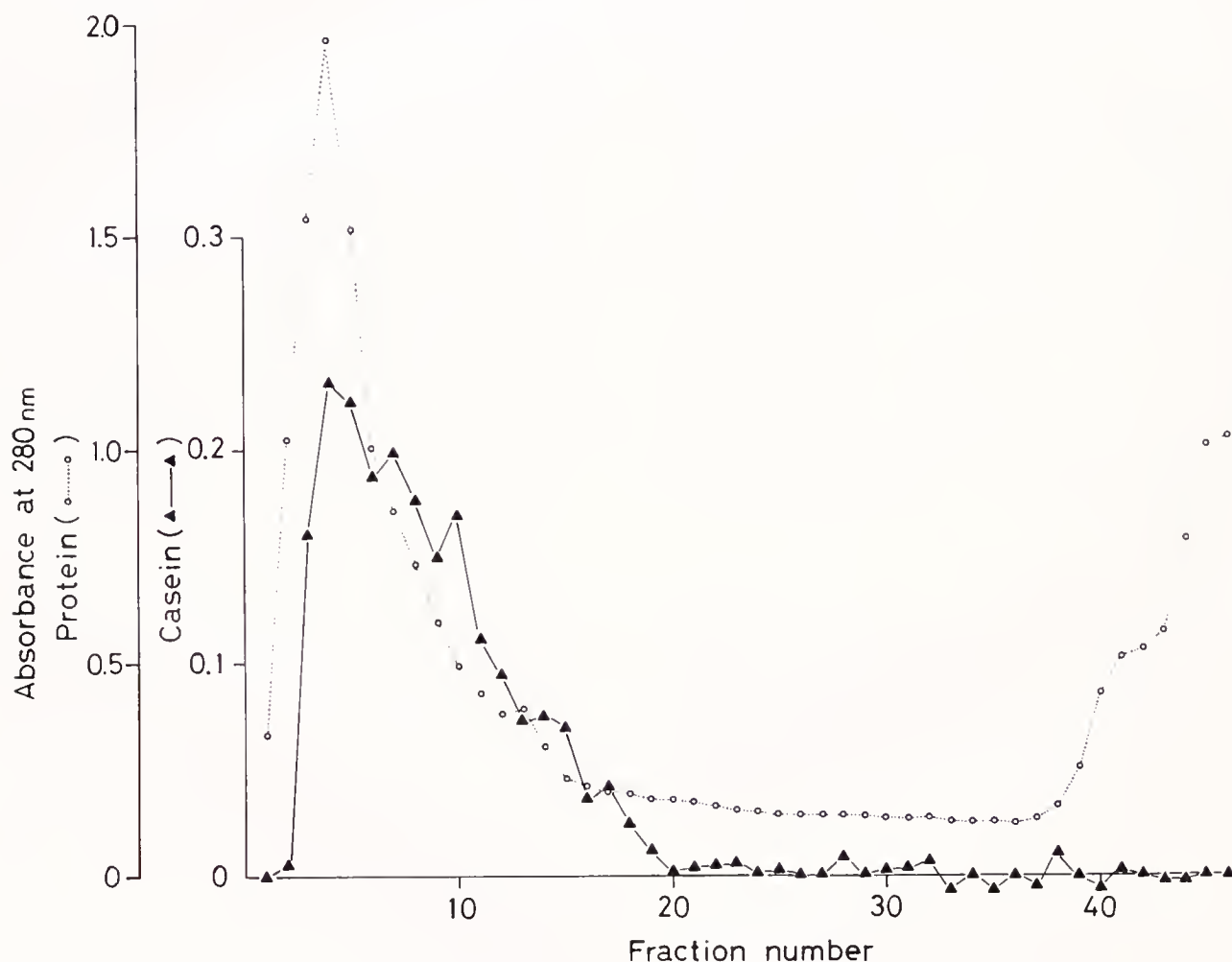


Figure 1. Elution pattern of the proteolytic activity on gel filtration chromatography. A 1.5 ml volume of concentrated hatch water (about 5 females) was adjusted to a Sephacryl S-200 column previously equilibrated with 10 mM Tris-HCl buffer (pH 8.5). Casein solutions (1%) were incubated for 30 min at 30°C with each fraction, and the absorbance of the deproteinized supernatant was measured at 280 nm (○...○). Absorbance of protein (▲—▲).

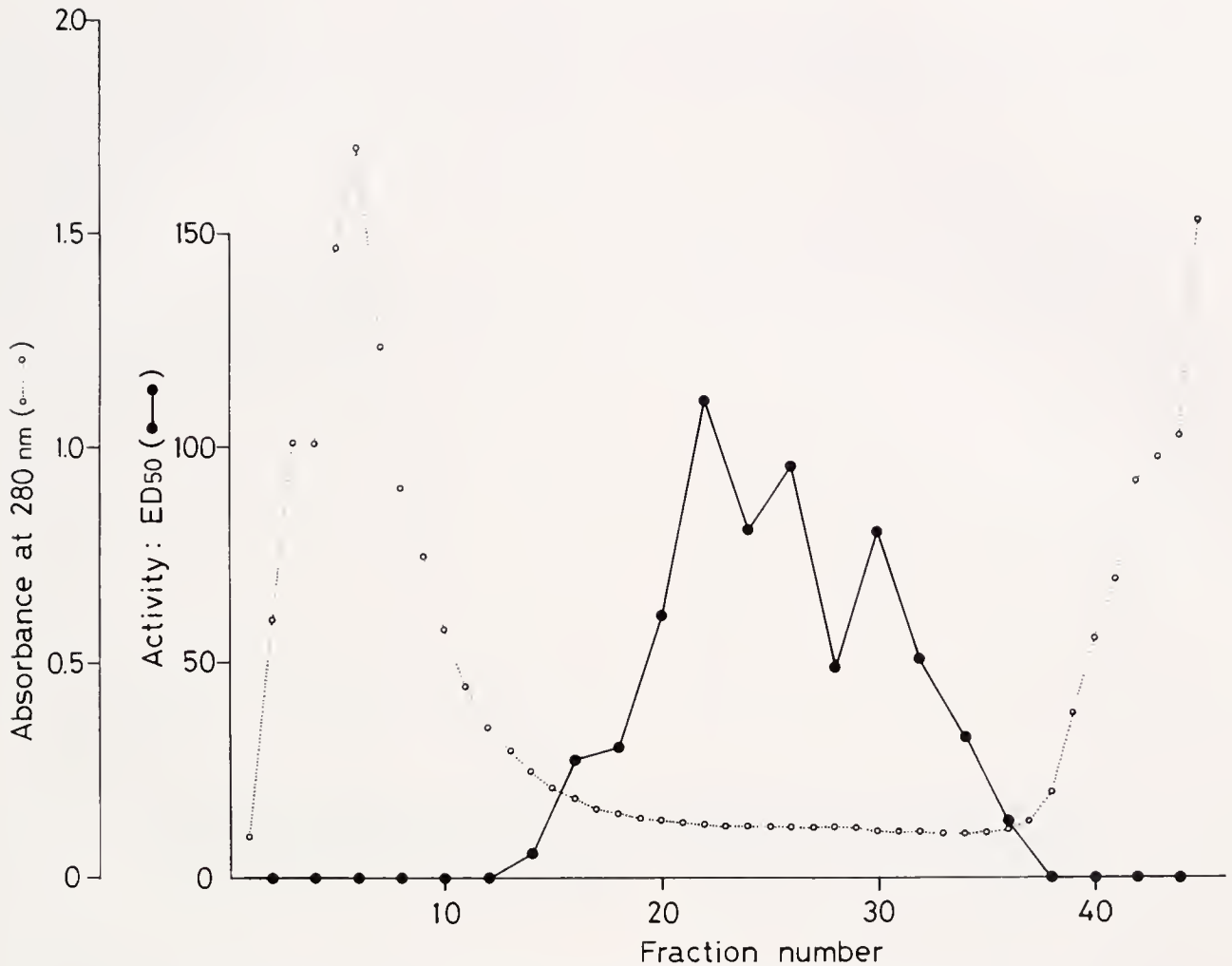


Figure 2. Elution pattern of OHSS activity on gel filtration chromatography. Concentrated hatch water (1.5 ml; 5 females) was subjected to the same gel filtration protocol shown in Figure 1. Clusters of embryos, freshly detached from three females, were incubated with each fraction for 2 h at about 25°C. OHSS activity (●—●); Absorbance of protein (○·····○).

proteinase occurs widely in intertidal and estuarine crabs (data not shown).

Elution of OHSS activity

Concentrated hatch water (1.5 ml; about five females) was subjected to the same gel filtration protocol used in the experiment of Figure 1. The activity of each fraction was assayed with unhatched embryos of *Sesarma haematocheir* for 2 hours (Fig. 2). The activity of OHSS extends over a wide range of fractions. As shown elsewhere (Saigusa, 1995), the molecular size of OHSS was estimated to be 15–20 kDa by a comparison of its elution volume with those of the standard proteins.

The pattern of OHSS activity on gel filtration (Fig. 2) is clearly different from that of proteinase (Fig. 1). There-

fore, at least two kinds of active factors are contained in crab hatch water, and both factors occur widely in intertidal and estuarine crabs.

Assay with egg-capsule debris

I next asked whether the hatch water would dissolve the egg capsule, and if so, whether OHSS and proteinase could be responsible for the activity. The assay was therefore performed with the powdered debris of the egg capsule as substrate. Hatch water (1.5 ml; about five females) that had been lyophilized and suspended in 10 mM Tris-HCl buffer (pH 8.5) was subjected to the same gel filtration protocol used in the experiments shown in Figures 1 and 2.

The debris of the egg capsule was dissolved by the

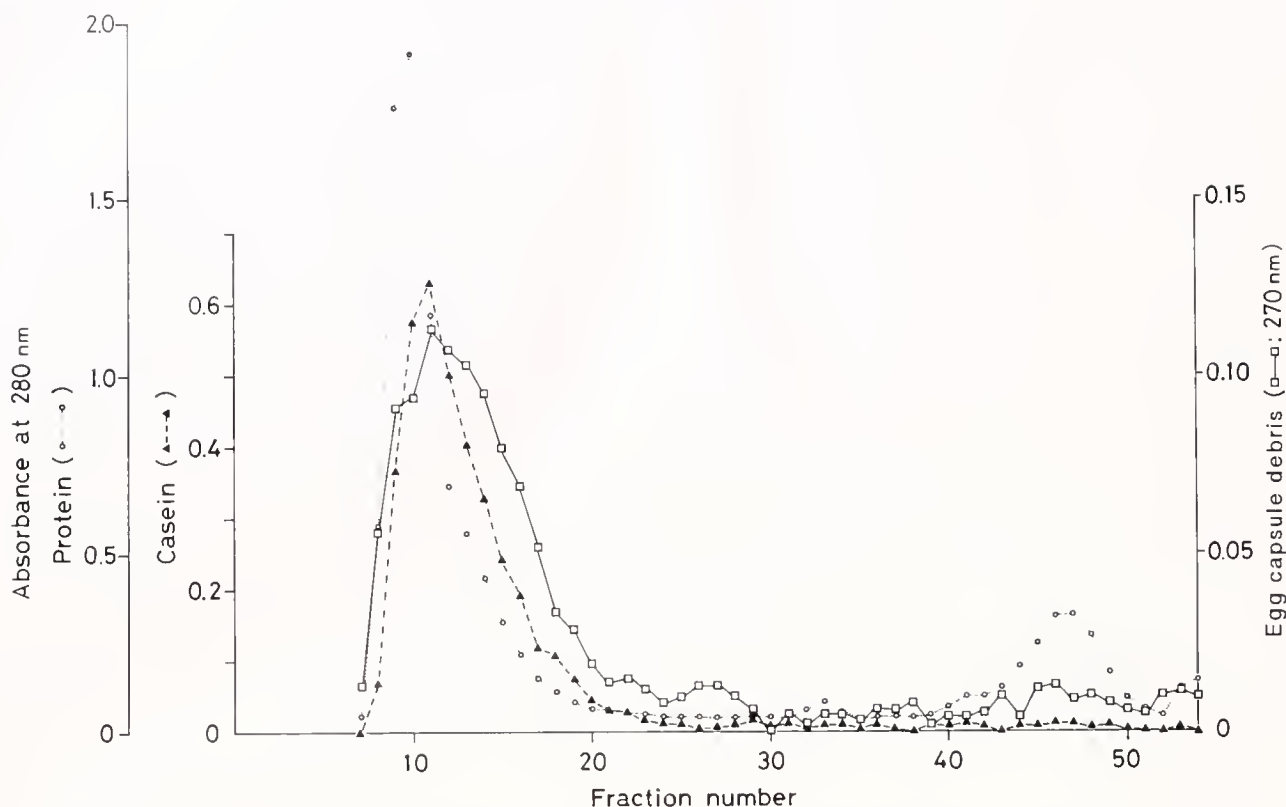


Figure 3. Dissolution of egg capsule debris by the proteinase. Concentrated hatch water (1.5 ml; 5 females) was subjected to the same gel filtration chromatography shown in Figure 1. The substrate solutions containing egg capsule debris or 1% casein were assayed with each fraction. Both solutions were incubated for 1.5 h at 30°C. Absorbance of protein (○·····○); proteolytic activity (▲---▲); digestion of egg-capsule debris (□—□).

hatch water (Fig. 3). The activity eluted as a single peak, and its pattern clearly agrees with that of the proteolytic activity (Fig. 1), and not with OHSS (Fig. 2). Thus the egg capsule debris is probably digested by the proteinase, but not by OHSS.

Some other experiments

To determine whether a chitinase is contained in the hatch water, degradation of colloidal chitin (*i.e.*, a homopolymer of β -linked *N*-acetylglucosamine) was examined. The assay method followed was that of Bade and Stinson (1979). In addition, the release of *p*-nitrophenol from 0.1 mM *p*-nitrophenyl 2-acetoamide-2-deoxy- β -D-glucopyranoside (*p*NP- β -GlcNAc) solution. The assay method was that of Dziadik-Turner *et al.* (1981). Neither *N*-acetylglucosamine nor *p*-nitrophenol was released from these substrates, suggesting that a chitinase is not contained in crab hatch water (data not shown).

Proteolytic activity was also assayed with an amidase substrate, *i.e.*, *N*-benzoyl-L-valylglycyl-L-arginine-*p*-ni-

troanilide (BValGlyArgNA); the method was that of Grant *et al.* (1981). BValGlyArgNA was dissolved in 50 mM Tris-HCl buffer (pH 8.5) to a concentration of 1 mg/ml (1.7 mM). The solution was further diluted 20 times with 100 mM Tris-HCl buffer (pH 8.5). The assay mixture contained 200 μ l of this diluted substrate solution and 200 μ l of the enzyme solution. The amount of *p*-nitroanilide liberated was monitored continuously at 385 nm for 4–5 min. Concentrated hatch water (1.5 ml; five females) was subjected to the same gel filtration protocol used in Figures 1 and 2.

This substrate (*i.e.*, BValGlyArgNA) was also decomposed by hatch water. The pattern of this activity on gel filtration was the same as that shown in Figure 2, suggesting that BValGlyArgNA is decomposed by the proteinase, not by OHSS (data not shown).

Discussion

This study indicates that the hatch water of the estuarine terrestrial crab *Sesarma haematocheir* contains a

proteolytic enzyme (Fig. 1) in addition to OHSS (*i.e.*, ovigerous-hair stripping substance) (Fig. 2). Both factors were separated by gel filtration. The proteinase dissolved the egg capsule debris, but the absorbance at 270 nm was very low, even at its peak, compared with the dissolution of casein (Fig. 3). This result raises a question about the function of the proteolytic activity: what part of the egg capsule is dissolved by this enzyme?

The embryos of crustaceans are encased in capsules comprising at least two distinct layers as observed with the stereomicroscope, although they may consist of several layers when examined at higher magnifications (Cheung, 1966; Goudeau and Lachaise, 1980). But the principal components are the outer layer, which is thick and tough, and the inner layer, which is very thin (Yonge, 1937, 1946; Marshall and Orr, 1954; Davis, 1959, 1964, 1965; and Anderson and Rossiter, 1969). A number of studies have suggested that the outer thick layer is not dissolved, but that it cracks when hatching occurs (for a review, see Davis, 1981). Nevertheless, a proteinase is released outside of the egg capsule at the time of hatching (Fig. 2), and it dissolves the debris of the egg capsule (Fig. 3). So where does the proteinase act?

In addition to these two layers, embryos of decapod crustaceans are invested by a transparent layer consisting of very sticky material. The existence of this layer becomes clear at the time of hatching, when the zoeas try to leave the broken egg capsule. This structure protrudes from the broken egg case upon the escape of the zoea (see fig. 3C in Saigusa, 1993). The sticky nature of this material is apparent when it is picked out with a forceps. The hatched zoeas escape from this layer by vigorous vibrating movements of their abdomen and limbs. Observations with a scanning electron microscope show this layer to be very thin and irregular in structure (see fig. 5b in Saigusa, 1992b).

For the experiment shown in Figure 3, the isolated egg capsules were washed repeatedly with tap water until the yolk was completely gone. Nevertheless, a small quantity of the fragments of embryonic tissues might have remained. Although the notion that those fragments is digested still remains, I suppose that the proteinase hydrolyzes this sticky layer upon hatching. This layer is very thin in the egg capsule, so the absorbance at 270 nm would have been very low. Yet, it is doubtful that such a small-scale dissolution of the egg capsule (Fig. 3) directly causes a rupture of the tough egg capsule of crustaceans.

Acknowledgments

Gel filtration chromatography was done at Ushimado Marine Laboratory, Okayama University. I thank Dr. Tadashi Akiyama for technical assistance. Supported by

Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture, No. 06839017 and No. 08833009 (Marine Biology).

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Ultrastructure and Transport-Related Enzymes of the Gills and Coxal Gland of the Horseshoe Crab *Limulus polyphemus*

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Abstract. The horseshoe crab, *Limulus polyphemus*, may be unique among marine arthropods in that both its book gills and its coxal gland may serve as sites of ion transport. We have therefore examined the ultrastructure of these organs, as well as the distribution and relative levels of two major transport-related enzymes: the $\text{Na}^+ + \text{K}^+$ ATPase and carbonic anhydrase (CA). The ventral surface of the central region of each lamella shows the typical ultrastructural specializations for ion transport: 10 μm cell thickness, an extensive network of tubules originating from infoldings of the basal membrane, and a high density of mitochondria. This region also contains high levels of activity of the $\text{Na}^+ + \text{K}^+$ ATPase and CA. The distribution of ion transporting epithelium and transport enzymes is identical in each of the five gill books. The peripheral region of the lamellae of each gill book is specialized for passive gas exchange. The ultrastructural and biochemical profile of the coxal gland is similar to that of the central-ventral region of the gill. *Limulus* possesses the same general mechanism of ion regulation seen in euryhaline decapod crustaceans, but the structural and functional components are uniquely distributed.

Introduction

Euryhaline invertebrates possess a full suite of adaptations for survival in and exploitation of habitats of low

and fluctuating salinity. While these adaptations are no doubt common to some extent in all euryhaline marine arthropods, they have been most extensively studied in the decapod crustaceans (*e.g.*, Mantel and Farmer, 1983). Considerably less is known about another arthropod group, the Xiphosura, of which the horseshoe crab, *Limulus polyphemus*, is a member. This investigation focuses on the ultrastructural and biochemical bases of salinity adaptation in *Limulus* as they compare to those in the ecologically similar but more highly derived decapods.

At high salinities, subtidal crustaceans are typical osmotic and ionic conformers. Below a critical salinity, however, many species have the ability to regulate hemolymph osmotic and ionic concentrations above those in the ambient medium. Strong regulators can maintain as much as a 600 mOsmol $\text{KgH}_2\text{O}^{-1}$ difference between the hemolymph and the surrounding water.

Ion regulation in decapod crustaceans is accomplished primarily through the active uptake of Na and Cl by the gill (*e.g.*, Cameron, 1978). The site of ion transport within the gill is the chloride cell (Foskett and Scheffey, 1982). This cell type is characterized in general as being mitochondria-rich and having an extensive network of infoldings of the basal membrane (Copeland, 1964; Copeland and Fitzjarrell, 1968; Oschman and Berridge, 1971; Cioffi, 1984). In addition, epithelia that are rich in chloride cells possess high levels of activity of two ion transport-related enzymes, the $\text{Na}^+ + \text{K}^+$ ATPase and carbonic anhydrase (CA) (Henry, 1984; Towle, 1984).

Chloride cells are heterogeneously distributed in the gills of euryhaline marine decapod crustaceans; they are

Received 1 November 1995; accepted 24 May 1996.

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more abundant in the posterior gills, especially at low salinities where they form a visible patch in the lamellae (Neufeld *et al.*, 1980; Compere *et al.*, 1989). Consequently, the posterior gills have been characterized as the site of ion regulation, containing dense patches of chloride cells and the associated high levels of transport-related enzymes; the anterior gills are believed to be more specialized for respiratory gas exchange.

The other potential transport epithelium, the antennal gland, is not believed to function in monovalent ion (*i.e.*, NaCl) regulation in euryhaline marine crabs. It produces an isosmotic and isoionic urine at high and low salinities, and it lacks comparable levels of CA activity found in the gill (Cameron, 1978; Cameron and Batterton, 1978; Henry and Cameron, 1982a). As a result, the antennal gland is thought to function in divalent ion regulation and to rid the extracellular compartment of excess fluid, with Na and Cl being lost in the bulk flow.

The horseshoe crab, *Limulus polyphemus*, is a marine chelicerate that is also euryhaline. Although much of its adult life is spent in offshore waters of high salinity (35 ‰), adult *Limulus* invades and survives in estuarine waters as low as 7 ‰ during its annual spawning migration (McManus, 1969; Cavanaugh, 1975; Cohen and Brockman, 1983). Characterized as a relatively weak osmotic regulator, the horseshoe crab maintains the osmotic concentration of its hemolymph roughly between 150 and 200 mOsm KgH₂O⁻¹ above ambient in low salinity (Robertson, 1970; Towle *et al.*, 1982, Mangum *et al.*, 1986). Although the gills of *Limulus* are not homologous to those of other marine arthropods (*e.g.*, crustaceans), they are functionally analogous. As in other marine arthropods, *Limulus* gills are morphologically differentiated into thin (peripheral) and thick (central) regions that, at least superficially (*i.e.*, gross morphology and tissue thickness), resemble the subdivisions of the respiratory and ion transporting regions, respectively, of crustacean gills. In *Limulus*, however, which possesses typical chelicerate book gills, the thick region is located centrally on each gill lamella, and the thin region is peripheral (Mangum, 1982).

On the other hand, the coxal gland of *Limulus*, which is homologous to the antennal gland of crustaceans, does not appear to be completely analogous in function. In *Limulus* acclimated to low salinity, the coxal gland produces urine that is hypoosmotic and hypoionic to the hemolymph (Mangum *et al.*, 1976; Towle *et al.*, 1982). Both the gills and coxal gland contain measurable levels of Na⁺ + K⁺ ATPase activity (Towle *et al.*, 1982). Therefore, both organs may play an important role in ion regulation. If so, then *Limulus*—one of the few large euryhaline arthropods with a reproductive and life history strategy linked to migration along a salinity gradient—

would be set apart from euryhaline decapods in its strategy of low salinity adaptation.

In the present investigation we have compared the differences between two phylogenetically distant, but ecologically similar groups. We present data on the ultrastructural features of the gills and coxal gland that are characteristic of salt-transporting epithelia. The activities of the Na⁺ + K⁺ ATPase and CA in gills and coxal gland were also compared.

Materials and Methods

Collection and maintenance of experimental animals

Adult horseshoe crabs, *Limulus polyphemus* (>20 cm carapace width), were collected from three locations: (1) an oceanside beach in Accomack County on the Eastern Shore of Virginia, (2) the Hampton Roads area of the Chesapeake Bay, and (3) the Gulf of Mexico near Port St. Joe, Florida. Animals were maintained either in running seawater tables (29–32 ‰ salinity) at the Virginia Institute of Marine Science, Eastern Shore Laboratory, Wachapreague, Virginia, or in 25–35 gallon plastic tanks, containing seawater of similar salinity, and equipped with biological filters for long-term acclimation in Williamsburg, Virginia, or Auburn, Alabama.

Animals were acclimated to high salinity (32 ‰) for three weeks. For acclimation to 10 ‰, salinity was decreased slowly (3–5 ‰ per day), and the animals were then held at 10 ‰ for at least two weeks before use. Salinity was measured either with a conductivity meter (Yellow Springs Inst. model 33) or a hand-held refractometer and adjusted with either artificial sea salt or deionized water. The horseshoe crabs were fed fish and shrimp three times per week but were starved for a period of no longer than one week prior to use in an experiment.

Gill ultrastructure

Lamellae from horseshoe crabs acclimated to either high (32–35 ‰) or low (15–18 ‰) salinity were removed from the gills and immediately placed in fixative (2% glutaraldehyde, 150 mM sucrose, 100 mM phosphate buffer, pH of 7.2–7.4). Small (2 × 4 mm) rectangular pieces of tissue were cut from both the central and peripheral regions of the lamellae. The ventral and dorsal chitinous layers were then separated, producing four sections: central dorsal (CD), central ventral (CV), peripheral dorsal (PD), and peripheral ventral (PV) (see Fig. 1 for a schematic of the dissection). Gill sections were fixed in fresh glutaraldehyde solution for 2 h followed by three 10-min rinses in phosphate buffer. Tissues were then post-fixed for 2 h in 1% osmium tetroxide in phosphate buffer and serially dehydrated with acetone. The gill tis-

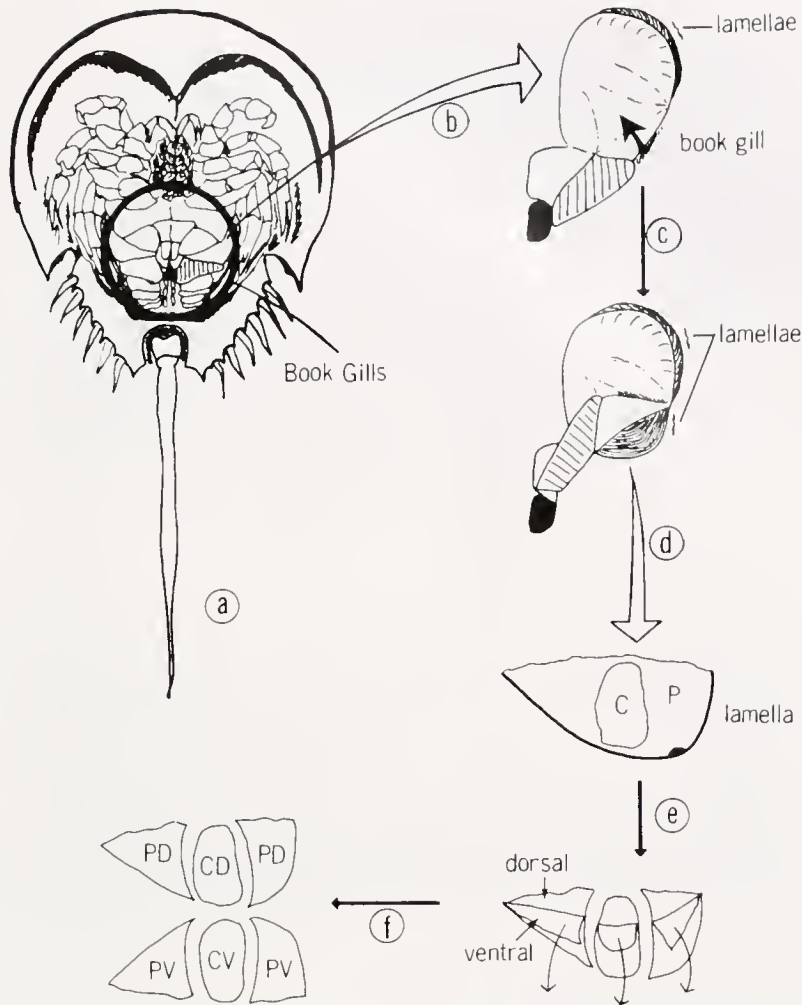


Figure 1. Procedure for dissecting the gill lamellae to produce standardized sections of tissue for electron microscopy and $\text{Na}^+ + \text{K}^+$ ATPase assays. (a) Ventral view of adult male horseshoe crab; (b) removal of gill book number 1; (c) lifting flap to expose lamellae; (d) removal of individual lamella; (e) removal of thick chitinous edge and dissection of the central from the peripheral regions; (f) separation of ventral from dorsal sides. Resulting regions: PD = peripheral dorsal, PV = peripheral ventral, CD = central dorsal, CV = central ventral.

sue was stained with uranyl acetate for 8–24 h and embedded in either SPURRS or EMBED medium. Thin sections, cut on a Sorvall MT 2-B ultramicrotome, were stained with lead citrate and examined with a Zeiss EM 9S-2 or EM 109 electron microscope.

$\text{Na}^+ + \text{K}^+$ ATPase activity

Upon the completion of salinity acclimation, all five of the gill books were removed from the animal along with a lobe of the coxal gland. The tissues were placed in about 4 volumes of cold homogenizing medium (0.25 M sucrose, 6 mM disodium EDTA, 20 M imidazole, pH adjusted to 6.8 with acetic acid) (Towle *et al.*, 1982). The

central dark patch of tissue was separated from the peripheral region in about 20 lamellae from each gill, and a sample of intact lamellae was taken as well. Tissues were transferred to 20 volumes of fresh homogenization medium containing 0.2 volumes of 10% sodium deoxycholate and homogenized by hand in a ground glass tissue grinder. The homogenate was filtered through two layers of cheesecloth and assayed immediately. Tissue samples from the coxal gland were dissected and homogenized as above without any attempt at separation into anatomically distinct areas.

Total ATPase activity was measured using a PK/LDH linked spectrophotometric assay described by Towle *et al.* (1982). Briefly, the assay measures the oxidation of

NADH at 340 nm that is enzymatically coupled to the hydrolysis of ATP. The reaction mixture contained 0.1 mM NADH, 5 mM disodium ATP, 2.5 mM PEP, 20 μ l PK/LDH, 20 mM imidazole (pH 7.8), 90 mM NaCl, 10 mM KCl, and 5 mM $MgCl_2$ in a final volume of 2.0 ml (all chemicals obtained from Sigma, St. Louis, Missouri). After 5 min incubation at 30°C, the reaction was started by the addition of up to 100 μ l of filtered homogenate. The change in absorbance was monitored in a temperature-controlled cuvette (30°C) and Bausch and Lomb 700, Beckman DU2 or DK2 recording spectrophotometer. Duplicate assays were run in the presence of 1 mM ouabain. The $Na^+ + K^+$ ATPase activity was taken as the difference between the total ATPase activity and the ouabain-sensitive ATPase activity. Total protein concentrations were determined by Coomassie blue dye binding (Bradford, 1976), using dye reagent (Bio Rad) and bovine serum albumin as a standard. Enzyme activity was reported as nanomoles Pi released per mg protein per minute. A two-way ANOVA followed by *a posteriori* testing (LSD) was used to determine significance in enzyme activities among the different gills and between the central and peripheral regions within the gills.

Carbonic anhydrase activity

Gill (central, peripheral, and whole sections) and coxal gland tissue were dissected as above from horseshoe crabs that had been acclimated to either 32 or 10 ‰ salinity. Tissues were placed in five volumes of cold buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris phosphate, pH adjusted to 7.4 with 10% phosphoric acid) and homogenized with a motor-driven ground glass homogenizer. The homogenate was centrifuged at $10,000 \times g$ for 20 min at 4°C (Sorvall RC-5B), and the supernatant was assayed for CA activity according to an electrometric method described by Henry (1991). Briefly, 100 μ l of CO_2 -saturated water is added to a reaction mixture containing supernatant and buffer (see above); the initial decrease in pH, due to the rapid hydration of CO_2 , is monitored using sensitive pH electrodes and a null-point pH meter. Since the conversion of CO_2 to H^+ has a 1:1 stoichiometry, the change in pH is representative of the rate of CO_2 hydration. Protein concentrations were determined by Coomassie blue dye binding (see above), and CA activities were reported as μ mol CO_2 per mg protein per minute. Statistical significance among the anatomical sections of the different gills, or among the whole-gill homogenates, at a given salinity was determined by ANOVA; significance within a single gill at high vs. low salinity was measured with Student's *t*-test. A portion of the gonadal operculum, anterior to the gills, was also assayed as a non-epithelial, salinity-insensitive "control" tissue.

Results

Gill and coxal gland ultrastructure

Each page, or lamella, of a book gill contains a thick, dark elliptical patch near its center surrounded by a thinner peripheral region (Fig. 2A). The central patch is similar in appearance to the dark, ion transporting region in the posterior gills of euryhaline crustaceans (*e.g.*, *Callinectes sapidus*; see Neufeld *et al.*, 1980). A longitudinal section through the central region is shown in Figure 2B. A network of pillar cells form channels in the lamellae through which hemolymph is moved; these pillar cells resemble those found in crustacean gill lamellae (Ciolfi, 1984). Hemolymph is separated from ambient water by a single epithelial layer on the dorsal and ventral surfaces of the lamellae. The thickness of the epithelium on the central ventral surface (Table I) is typical of ion-transporting epithelia found in gill lamellae of many crustacean species (*e.g.*, Aldridge and Cameron, 1979; see also Henry, 1994, for a recent review). The difference in epithelial thickness between the central ventral and the other lamellar sections appears to be due to the cellular component, since the thickness of the cuticle is the same throughout the gill (Table I). On the dorsal surface the total diffusion distance is only slightly greater ($<1 \mu$ m) than the cuticle thickness, indicating the presence of a very thin, respiratory type of epithelium. The peripheral ventral region is only slightly thicker, still suggesting an essentially respiratory function.

In contrast, the epithelium from the ventral surface of the central region displays ultrastructural features that are characteristic of salt-transporting cells of arthropod gills. The cells contain an extensive internal network of invaginations, originating from infoldings of the basal membrane (Fig. 3). There is also a very dense population of mitochondria associated with the network of membrane invaginations. At the very apical end of the cell, the membrane network and associated mitochondria are much less dense. Each cell has a prominent nucleus that is located in the basal portion. All of these features are similar to those found in "chloride cells" of both arthropods and fish (*e.g.*, Zadunaisky, 1984). The characteristic features that appear to be lacking in the epithelial cells of the peripheral region are the extensive system of membrane invaginations, the dense population of mitochondria, and the thick cellular component of the epithelium (Fig. 4A vs. 4B). Additional details may be found in Jackson (1986).

The coxal gland appears to have the same ultrastructural characteristics correlated with salt transport that are found in the central ventral region of the gills. There is an extensive network of membrane invaginations

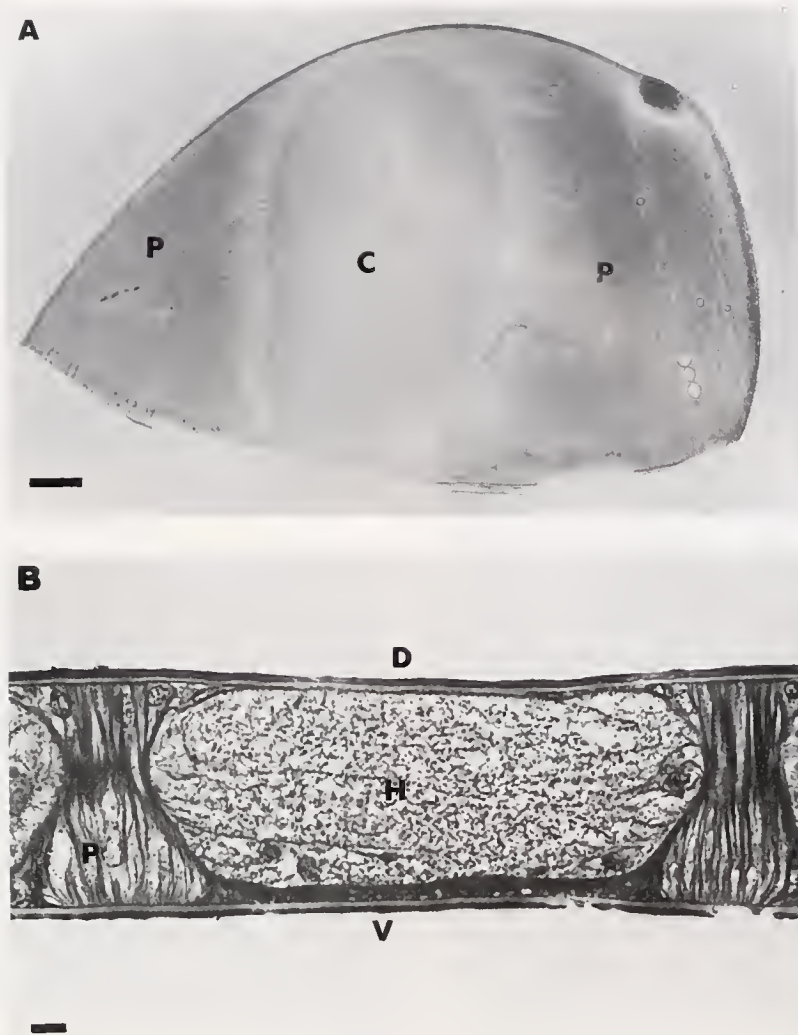


Figure 2. (A) Whole mount of a single lamella showing central (C) and peripheral (P) regions. $\times 4.5$, scale bar = 2 mm. (B) Longitudinal section through the central region showing the thin dorsal (D) and thick ventral (V) epithelial layers. P = supporting pillar cell network, H = hemolymph space. $\times 608$, scale bar = 10 μm .

Table I

Cuticular thickness and hemolymph-water diffusion differences in the various sections of horseshoe crab gills

Section	Cuticle thickness (μm)	Diffusion distance (μm)
CV	$3.7 \pm .10$ (18)	$9.1 \pm .24$ (14)
CD	$3.0 \pm .02$ (8)	$3.9 \pm .13$ (8)
PV	$3.4 \pm .08$ (20)	$4.7 \pm .31$ (10)
PD	$3.0 \pm .07$ (26)	$3.9 \pm .10$ (22)

Values reported as Mean $\pm$ SEM (N). CV = central ventral, CD = central dorsal, PV = peripheral ventral, PD = peripheral dorsal.

throughout the cells that is associated with numerous mitochondria (Fig. 5).

Na⁺ + K⁺ ATPase activity

For animals acclimated to 32 ppt salinity, whole-gill homogenates had ouabain-sensitive $\text{Na}^+ + \text{K}^+$ ATPase activities in the range of 35–65 nmol Pi mg pro⁻¹ min⁻¹; activity in the coxal gland was significantly higher at about 120 nmol Pi mg pro⁻¹ min⁻¹ (Fig. 6). Moreover, while activity among whole-gill homogenates was variable, the distinctive anterior-posterior differences in $\text{Na}^+ + \text{K}^+$ ATPase activity seen in crustacean gills were clearly absent. Gill numbers 2 and 3 had significantly



Figure 3. Semi-tangential section through a presumptive ion transporting cell in the ventral portion of the thick region. Note the elaborate membrane tubule network originating from infoldings of the basal membrane, the dense population of mitochondria, and the basal nucleus (n). Apical region of the cell is under the cuticle (c), and the basal region is in contact with the hemolymph (h). $\times 4515$, scale bar = $1\ \mu\text{m}$.

higher activities than gills 1, 4, and 5. The values for the gills were about 2–3 fold higher than those reported by Towle *et al.* (1982), while activity in the coxal gland was 30% lower than reported previously for animals acclimated to comparable salinities.

The distribution of the $\text{Na}^+ + \text{K}^+$ ATPase activity within each individual gill, however, was highly localized. The central section of the lamellae contained the majority of the enzyme activity. $\text{Na}^+ + \text{K}^+$ ATPase activity in the central region (approximately $100\ \text{nmol Pi mg pro}^{-1}\ \text{min}^{-1}$) was about eightfold greater than that in the peripheral region (Fig. 6). With the exception of gill number five, there was no significant difference in $\text{Na}^+ + \text{K}^+$ ATPase activity within the central region in anterior vs. posterior gills. The relatively high variability of $\text{Na}^+ + \text{K}^+$ ATPase activity in whole-gill homogenates (vs. relatively low variability in the central region) might reflect the variability, from gill to gill, in the ratio of the mass of the central region to that of the whole gill. Activity in the peripheral region, however, was uniformly low through-

out all gills. Further physiological differentiation was revealed when the dorsal and ventral epithelia were peeled apart and the individual layers examined. In the central but not the peripheral regions, $\text{Na}^+ + \text{K}^+$ ATPase activity was higher in the ventral portion of the epithelium. Acclimation to low salinity (15 ‰) for 36 h did not result in any increase in enzyme activity in either the gills or the coxal gland (data not shown); lack of short-term $\text{Na}^+ + \text{K}^+$ ATPase activation in *Limulus* gills was also reported by Towle *et al.* (1982).

Carbonic anhydrase activity

The distribution of CA in *Limulus* gills followed that of the Na/K ATPase, the pattern in most arthropod ion

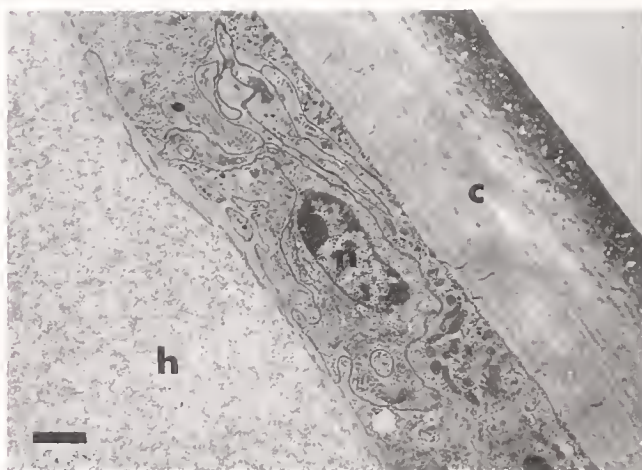
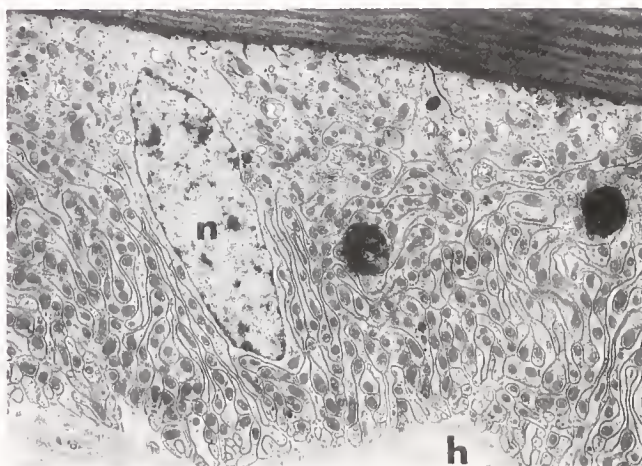


Figure 4. Comparison between presumptive ion transporting cells of the central ventral region of the gill (top) and presumptive respiratory epithelial cells of the peripheral region (bottom). The peripheral region lacks the extensive internal tubular system and the high density of mitochondria. $\times 7500$, scale bar = $1\ \mu\text{m}$.

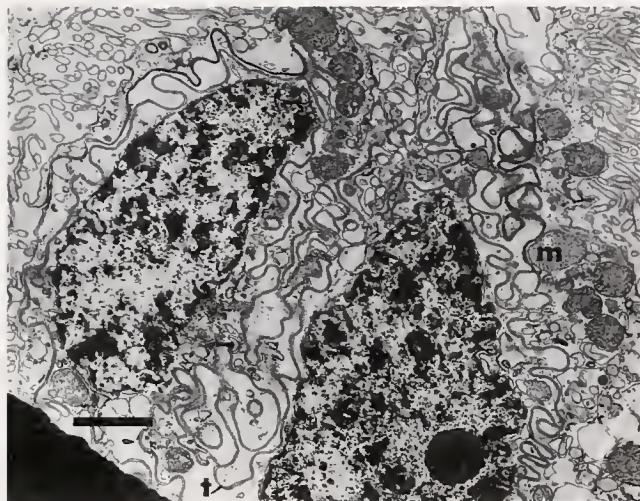


Figure 5. Tangential section through presumptive ion transporting cells of the coxal gland. Note the extensive internal tubular system (t) and numerous mitochondria (m). $\times 14,400$, scale bar = $1\ \mu\text{m}$.

transport epithelia. In animals acclimated to 32 ‰, the level of CA activity among the five gill books was uniform (Fig. 7A). The uniform distribution and levels of activity ($200\text{--}300\ \mu\text{mol CO}_2\ \text{mg pro}^{-1}\ \text{min}^{-1}$) were both similar to those seen in euryhaline marine crustaceans (e.g., Henry and Cameron, 1982a; Piller *et al.*, 1995). The central region of the lamellae also contained the bulk of the branchial CA activity; levels there were be-

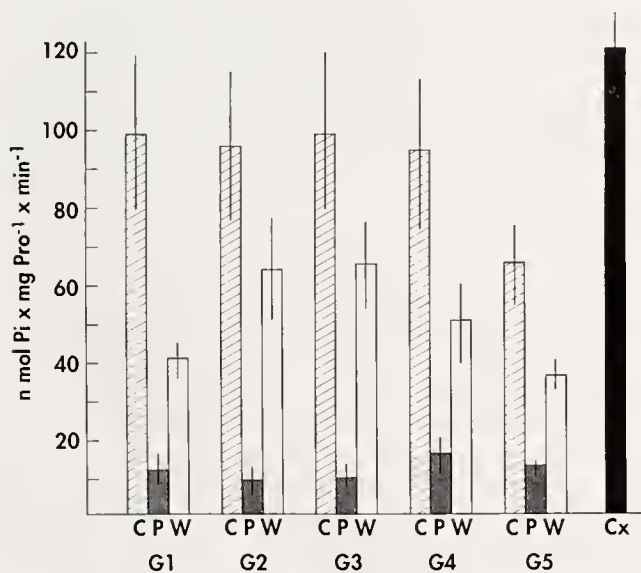


Figure 6. $\text{Na}^+ + \text{K}^+$ ATPase activity in homogenates of the five gill books (G1–5) and coxal gland (Cx) of *Limulus* acclimated to 32 ‰ salinity. Data for the gills are divided into whole-gill activity (W), and activity from the central (C) and peripheral (P) regions. Mean $\pm$ SEM ($n = 5\text{--}6$).

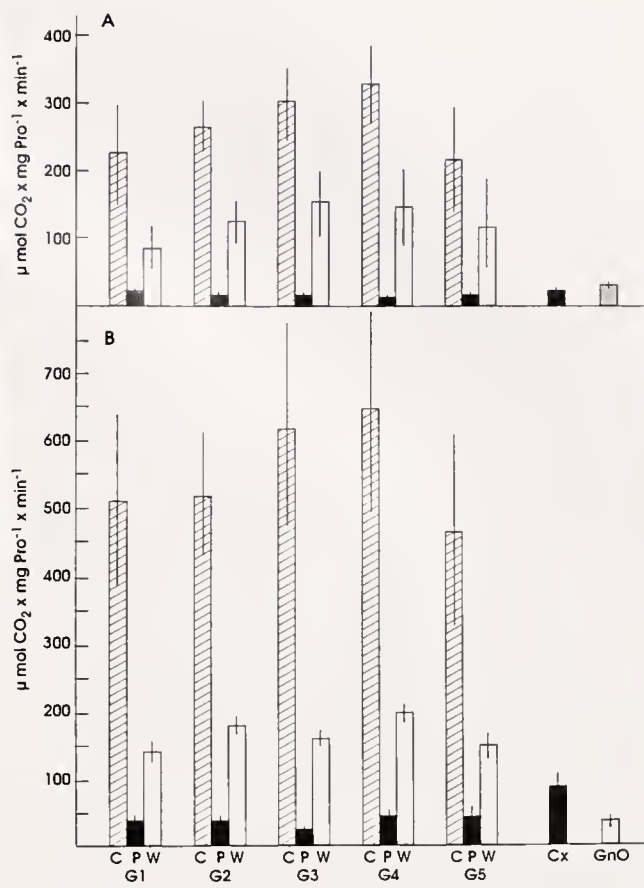


Figure 7. Carbonic anhydrase activity from the five gill books (G1–5), coxal gland (Cx) and gonadal operculum (GnO) of *Limulus* acclimated to 32 ‰ (A) and 10 ‰ (B) salinity. Gill activity is divided into whole-gill activity (W), and activity from the central (C) and peripheral (P) regions. Mean $\pm$ SEM, $n = 6$.

tween 10 and 20 times greater than those in the peripheral region. Unlike the $\text{Na}^+ + \text{K}^+$ ATPase, however, CA activity in the coxal gland was low compared to that in the gill (Fig. 7A).

Long-term acclimation (2 weeks) to 10 ‰ salinity induced CA activity in all gills and in the coxal gland (Fig. 7B). Activity in whole-gill homogenates increased from 5 to 65% with virtually all of the induced CA activity occurring in the central region of the lamellae. While the CA activity in the peripheral region increased significantly, the absolute values were very small (e.g., an increase of $12\ \mu\text{mol CO}_2\ \text{mg pro}^{-1}\ \text{min}^{-1}$ for gill 3). By comparison, the increases in the central regions of all gill books were very large (250 to $300\ \mu\text{mol CO}_2\ \text{mg pro}^{-1}\ \text{min}^{-1}$). After low salinity acclimation, CA activity in whole gills doubled at best, and the increase occurred uniformly in all gills. CA activity in the coxal gland tripled in animals acclimated to 10 ‰, but it remained low in comparison with the gills.

Discussion

Despite being an osmotic and ionic regulator of moderate capacity, *Limulus* has a migratory pattern that brings it into estuarine waters of low salinity and shares this feature with euryhaline decapods that are strong regulators. *Limulus* is typically found in nature in salinities as low as 7–9 ‰ (McManus, 1969; Cavanaugh, 1975; Cohen and Brockman, 1983), but it has also been reported to survive in 2 ‰ in the laboratory (Warren and Pierce, 1982). Depending on acclimation salinity, *Limulus* maintains its hemolymph between 50 and 300 mosm Kg H₂O⁻¹ above the ambient medium, which at best is only half the osmotic gradient that strong regulators (e.g., *C. sapidus*) can support. To survive in and exploit estuarine waters, *Limulus* appears to have independently evolved many of the same physiological and biochemical adaptations found in both euryhaline marine and freshwater arthropods.

Like many euryhaline and freshwater decapod crustaceans, the salt-transporting cells in *Limulus* are concentrated in a specific area within the gill lamellae; in this case it is the central region, and perhaps even more specifically the central ventral region of the lamellae of the book gills. In all species thus far examined, the region of the gill in which the salt-transporting cells (chloride cells) are localized also contains the highest levels of activity of both the Na-K ATPase and CA. *Limulus* differs from euryhaline decapods, however, in that both the distribution of chloride cells and corresponding enzyme activities are uniformly distributed among all gills. There is little or no anterior-posterior differentiation in the distribution of chloride cells, Na⁺ + K⁺ ATPase activity, and CA activity that is characteristic of other species (Aldridge and Cameron, 1979; Neufeld *et al.*, 1980; Henry and Cameron, 1982a; Holliday, 1985; Harris and Bayliss, 1988). In this way *Limulus* is more similar to freshwater crayfish, which also have a uniform distribution of both chloride cells (Dickson *et al.*, 1991) and ion transport-related enzyme activities (Wheatly and Henry, 1987). The gills of *Limulus* have only 10–50% of the Na/K ATPase activity as do those of strong ion regulators, such as *Callinectes sapidus* (and other decapod crustaceans; e.g., 100–400 nmol Pi mg pro⁻¹ min⁻¹) (Towle *et al.*, 1976; Neufeld *et al.*, 1980; Siebers *et al.*, 1982; Holliday, 1985). On the other hand, Na/K ATPase activity in the coxal gland is much higher than that in the antennal gland of both euryhaline marine and freshwater ion regulators (Towle, 1981; Wheatly and Henry, 1987).

Among euryhaline marine arthropods, *Limulus* appears to be unique in that its excretory organ, the coxal gland, has both the ultrastructural and biochemical characteristics of an ion transporting epithelium and also ap-

pears to play a clear role in hemolymph ion regulation (Mangum *et al.*, 1976; Towle *et al.*, 1982; also present findings). The present results show that CA activity is much higher than that found in the antennal gland of euryhaline decapods (e.g., Henry and Cameron, 1982a), and that it is sensitive to salinity. In contrast, low and salinity-insensitive CA activity in the antennal gland of euryhaline decapods is believed to reflect the organ's primary role in the elimination of excess water; in fact, urine produced by the antennal gland is a major route of salt (NaCl) loss (Cameron and Batterton, 1978; Wheatly, 1985). Furthermore, Na⁺ + K⁺ ATPase activity in the coxal gland of *Limulus* is higher than that reported for the antennal gland of a moderately euryhaline crayfish (Wheatly and Henry, 1987); the antennal gland functions in the active reabsorption of Na and Cl and thus plays an important role in hemolymph ion regulation (Wheatly and Toop, 1989).

CA activities in the central region of the *Limulus* gill and in the coxal gland are salinity-sensitive and are induced by exposure to low salinity. The time course of induction, about two weeks, is similar to that found in both euryhaline marine and freshwater decapods (Henry and Cameron, 1982b; Henry and Wheatly, 1988). The degree of induction (twofold) was relatively small compared to the 8- to 10-fold changes in CA activity seen in the posterior gills of euryhaline crustaceans that are strong ion regulators (Henry and Cameron, 1982a; Piller *et al.*, 1995). There was no change in Na⁺ + K⁺ ATPase activity in either the gills or the coxal gland over a 36-h transfer from high to low salinity. This was also reported by Towle *et al.* (1982) and suggests that short-term activation of existing enzyme was not taking place. Activity was not monitored for a longer period.

Given the apparent ion-regulatory nature of the central region of the gill and the coxal gland, we suggest that the lower limit of salinity tolerance in *Limulus* may not be set by an inability to take up and conserve ions. Rather, the high water permeability of both the carapace and gills, and the resultant tissue swelling that accompanies hemolymph dilution in low salinity, may be the constraints that restrict *Limulus* from invading and permanently inhabiting waters of low salinity (<10 ‰). The water permeability of the carapace of *Limulus* is about tenfold higher than that in decapod crustaceans (Hannan and Evans, 1973; Dunson, 1984). In addition, the gills in *Limulus* are estimated conservatively at being fivefold more permeable to water than the carapace, while permeability to salts is relatively low (Dunson, 1984). Severe swelling in the gills has been reported for *Limulus* in low salinity, and this has been correlated with ballooning and hemorrhage as a possible cause of death (Mangum *et al.*, 1976; Dunson, 1984). Water gain, and not salt loss, is

probably the physiologically disruptive event in low salinities.

High water and low salt permeability would tend to establish a steep osmotic gradient between hemolymph and water in low salinity, and this would result in significant water gain by tissues. Heart tissue wet weight increases about 140% immediately upon transfer to low salinity, and interestingly, intracellular volume readjustment is slow and incomplete (Warren and Pierce, 1982). *Limulus* lacks the typical mechanism of rapid cell volume readjustment through a reduction of the intracellular free amino acid pool (Moran and Pierce, 1984); rather it relies on a biphasic mechanism of early intracellular ion loss followed by a much slower reduction in intracellular glycine betaine concentrations (Warren and Pierce, 1982). At extremely low salinities, this could limit the ability of *Limulus* tissues to reduce swelling; or the loss of intracellular K^+ beyond a critical point could cause enough damage to excitable tissues such as nerve and muscle to result in systemic failure. Severely swollen gills have indeed been reported for *Limulus* in low salinity (Mangum *et al.*, 1976; Dunson, 1984).

High water permeability has been proposed as an adaptation for the reproductive strategy of *Limulus*, as these high permeability values originate in the early developmental (egg and globe) stages (Laughlin, 1981). The egg cases are deposited intertidally in sand beaches and are therefore potentially subject to desiccation. High water permeability would ensure rapid rehydration upon contact with water, while low ion permeability would ensure conservation of salts in the face of exposure to freshwater runoff. Limitations in ion regulation and cell volume readjustment in the adult may have resulted from the selection for mechanisms of ensuring a rapid and constant state of hydration in the early developmental stages.

Acknowledgments

This work was supported in part by NSF IBN 93-04844 to RPH, and by funds from the Alabama Agricultural Experiment Station.

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Reports of Papers Presented at the General Scientific Meetings of the Marine Biological Laboratory

August 12 to 14, 1996

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ABSTRACTS

In addition to the work described here in Short Reports, the following papers were also presented. The abstracts of these papers are available from the Marine Biological Laboratory Archives, 7 MBL Street, Woods Hole, MA 02543.

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Water turbidity limits *Limulus* visual performance
- Williams, T., A. Galione, F. Dubé, and W. R. Eckberg**
Mechanisms of calcium sequestration and release in the oocytes of *Chaetopterus pergamentaceus*

Introduction to Featured Article: Hearing and Communication Through Sound in Toadfish (*Opsanus tau*)

During courtship, male toadfish (Fig. 1A) produce the “boatwhistle.” This is a long, loud sound, consisting of a fundamental frequency (130–250 Hz) and its higher harmonics—and it is attractive to females. Our research program focuses on the saccule of the ear (Fig. 1B), and its mechanisms for detecting, recognizing, and localizing male vocalizations, as well as other sound sources.

The saccule (like all the sensory epithelia in the ear) is composed of hair cell mechanoreceptors. Each hair cell has a bundle of stereovillae of graded length and a single, eccentrically located kinocilium. The hair cells are structurally polarized in that they respond best when their stereovillae are deflected in a direction toward the kinocilium (Fig. 1C). The population of hair cells in each saccule is arranged with respect to their polarity such that a wide range of orientations, and thus oriented responses, is represented (Fig. 1D). Finally the saccule also contains an otolith—a calcium carbonate concretion—that restrains the tips of the stereovillae. Thus the toadfish saccule is an otolith endorgan, examples of which are widely distributed among the invertebrates and in all vertebrates. Sound passing through a toadfish causes its body to oscillate along the axis of acoustic particle motion, but the otolith tends to remain at rest due to its greater inertia. Therefore, a relative motion is set up between the otolith and the hair cells, resulting in a deflection of the stereovillae.

The sensory epithelia of the ear are innervated by afferents of the eighth cranial nerve. We have recorded from saccular afferents and have found that they respond to particle movement with the same sensitivity as the mammalian inner ear (0.1 nm). Most afferents have directional response patterns (Fig. 1E), indicating that they receive input from a group of hair cells having similar directional orientations. Each saccular afferent fiber has a characteristic axis of best sensitivity (Fig. 1F) that varies widely among neurons, in both

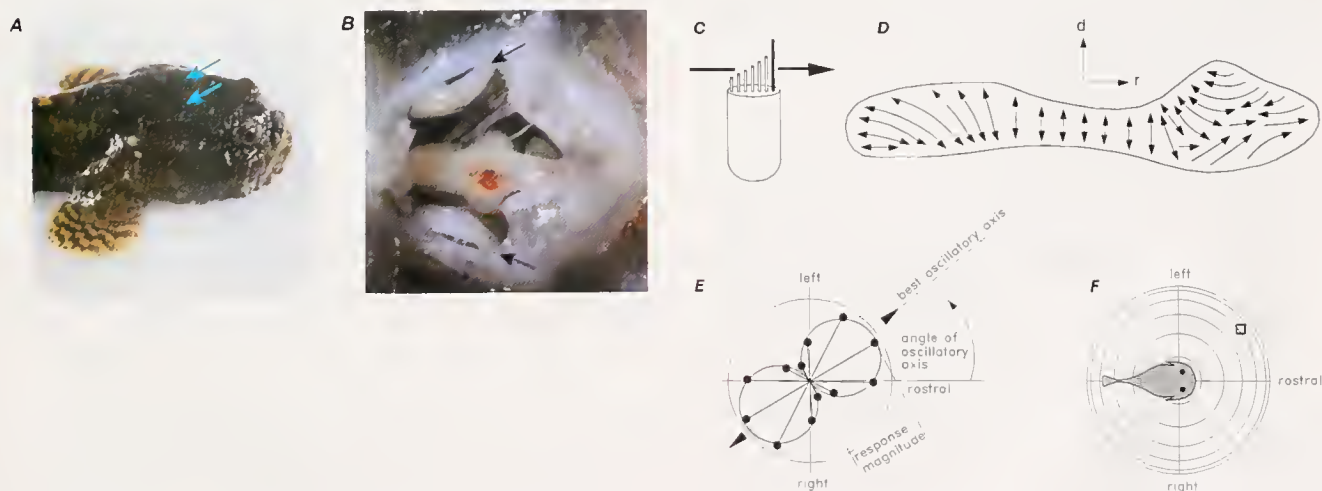


Figure 1. (A) Dorsal view of the toadfish (*Opsanus tau*) head illustrating the approximate location of the paired ears beneath the skull (blue arrows). (B) Ventral view of the toadfish braincase with the ears and brain exposed. The black arrows point to the two curved saccular otoliths. The otolith is 6 mm long. (C) Cartoon of a hair cell showing the eccentrically placed kinocilium (solid line) and the stereovillae. Deflection of the stereovillae toward the kinocilium (large arrow) is excitatory. (D) Schematic diagram showing the pattern of hair cell orientations on the saccular epithelium of the toadfish. The arrows represent the best direction for hair cells in that region. d = dorsal; r = rostral. (E) The magnitude of the response of an afferent neuron from the rostral region of the left saccule plotted, on polar coordinates, against the angle of the axis of oscillatory particle motion. Data are plotted twice, once at the nominal axis angle, and again at this angle plus 180° to indicate that the response magnitude is averaged over many cycles of oscillatory motion. The dashed line with arrowheads indicates the oscillatory axis that is most excitatory for this neuron. (F) Directionality of the afferent in E represented in spherical coordinates. This view is looking down on the north pole of a globe with the toadfish at its center. The square symbol marks the location in the northern hemisphere at which the most excitatory axis of the afferent penetrates the surface of the globe.

azimuth and elevation. These direction-selective signals to the brain carry information that could be used to compute the axis of acoustic particle motion and thus the direction of the sound source.

Most recently we have characterized the frequency range (<50–250 Hz) within which acoustic signals are encoded by the saccule. In general, saccular afferents are sufficiently sensitive and broadly tuned that they can encode the fundamental frequency of the boatwhistle, but not the higher harmonics. Thus, the information communicated by the voice of the male toadfish is probably the fundamental frequency, and this, in turn, is represented in the temporal patterns of neural activity received by the brain.

—Richard R. Fay, Peggy L. Edds-Walton, and Stephen M. Highstein
August 1996

Reference: *Biol. Bull.* **191**: 256–257. (October, 1996)

Tuning in Saccular Afferents of the Toadfish Revealed by the Reverse Correlation Method

Richard R. Fay¹, Peggy L. Edds-Walton², and Stephen M. Highstein³ (Marine Biological Laboratory)

Male toadfish (*Opsanus tau*) attract females with the “boat-whistle,” a long-duration, broad band vocalization composed of repeated pulses [130 pulses s⁻¹ in the Woods Hole area (1)] and produced at levels of 40 dB with respect to 1 dyne cm⁻² within a meter of the source (2). Acoustic particle motions of these sounds can be detected by the saccule, an otolithic endorgan having an auditory function in toadfish (3). Previous research (4) showed that primary saccular afferents respond in a directional manner to 100 Hz particle motions as small as 0.1 nm. To more fully understand the neurally coded representations of vocalizations, we used the reverse-correlation method (5,6) to determine the frequency filtering characteristics of saccular afferents.

As described previously (3), we recorded extracellularly from afferents while oscillating the toadfish in a water-filled cylinder driven by a three-dimensional shaker system. Stimuli were 20 random noise samples, 820 ms in duration, with flat acceleration magnitude-spectra between 50 and 1000 Hz. The noise samples were presented in each of three orthogonal axes (vertical, side-side, front-back).

Accelerometer recordings of the noise-driven motion of the cylinder were averaged for a period of 102.4 ms, *i.e.*, 51.2 ms before and after each spike. This averaged waveform estimates the impulse response of the linear filtering that precedes spike generation for phase-locked afferents (5). A fast-Fourier transform of the impulse response estimates an afferent's filter shape between 50 and 1000 Hz within a 20–30 dB dynamic range. Impulse responses were obtained for 98 afferents in 16 toadfish using several noise levels within the dynamic range.

Figure 1 shows impulse responses (right) and filter shapes (left) for eight representative afferents from two toadfish. Impulse responses are brief, damped oscillations indicative of

broadly tuned filters. For afferents having similar filter shapes, impulse responses often differ only in polarity (*e.g.*, compare R1 and R4). Polarity differences are probably due to signals from hair cell receptors that are oriented in opposite directions.

Filter shapes (Fig. 1, left), in general, have band-pass characteristics and differ primarily in high cut-off frequency (*i.e.*, in bandwidth) and slope (*e.g.*, compare R12 and R4). Filter shapes tend to be independent of stimulus level. The high-frequency point at which power falls by half ranges between 80 and 230 Hz. Afferents with high cut-off frequencies may (R12), or may not (S16), share a best frequency in the range between about 60 and 100 Hz with more narrowband afferents.

In general, saccular afferents of the toadfish encode the waveform of acoustic particle motion between <50 and 250 Hz. These and previous (3,4) results indicate that saccular afferents have a sufficiently high sensitivity and broad frequency response to encode the fundamental frequency component (*i.e.*, pulse repetition rate) of the boatwhistle within several meters from the source.

Supported by a Program Project Grant from NIH, NIDCD.

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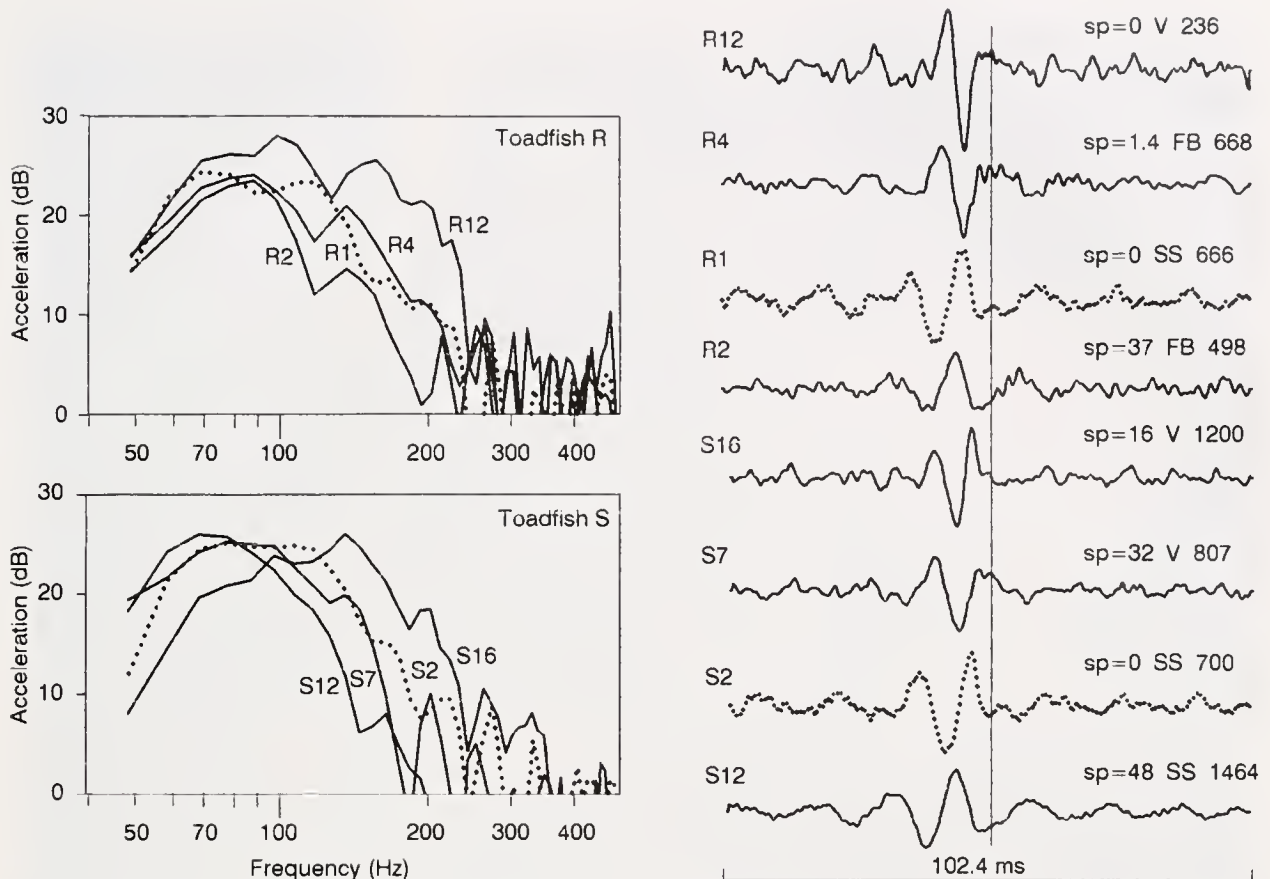


Figure 1. Left: Filter shapes for eight representative saccular afferents from two toadfish (R and S). The ordinate is acceleration in dB with an arbitrary reference. Right: Impulse responses (102.4 ms in duration) used to compute the filter shapes above. Vertical line through the impulse responses indicates the time of spikes. Dotted lines serve to help visually separate filter functions. Data shown were selected for the most excitatory stimulus direction and for a stimulus level near the middle of an afferent's dynamic range. At the right of each impulse response is given the spontaneous rate (sp) in spikes/s, the most excitatory direction (V = vertical, SS = side-side, FB = front-back), and the number of spikes used in constructing the impulse response.

Reference: *Biol. Bull.* **191**: 257–259, (October, 1996)

Segmental Organization of Vestibular and Reticular Projections to Spinal and Oculomotor Nuclei in the Zebrafish and Goldfish

Hiroshi Suwa (New York University Medical Center), Edwin Gilland, and Robert Baker

The developing hindbrain of vertebrates contains a series of eight neuromeric units called rhombomeres (1). These neuroepithelial compartments express restricted combinations of developmental genes that are believed to define the identity and histogenic fate of each hindbrain segment (2, 3). Because of their accessibility and simplicity, zebrafish embryos provided a model in which the segmental origin of reticular neurons projecting to the spinal cord were demonstrated for the first time (4). Even after the disappearance of recognizable rhombomeric boundaries, reticular neurons (5) retain their segmental organization, thus permitting recognition of rhombomeric segments (rhs) in the adult hindbrain of zebrafish (6) and goldfish (7).

To further characterize the spatial segregation of neuronal groups within the post-embryonic rhombomeric blueprint, we recon-

structed the hindbrain reticular and vestibular scaffold in juvenile goldfish and zebrafish with confocal microscopy after the placement of rhodamine-dextran crystals (3000 mw, Molecular Probes Inc.) in either the spinal cord at C2–4 or the medial longitudinal fasciculus (MLF) at the level of the IVth cranial nucleus. Tracer applications were limited to less than 1 min and transport times ranged from 1 to 3 h before perfusion with 4% paraformaldehyde. Whole hindbrains, cleared in glycerol, were scanned on either a Zeiss LSM-410 or a Leica TCS confocal microscope at 2–4 μ m Z-axis intervals. The locations of vestibular and reticular neurons were visualized in wholemount preparations as pseudocolored depth profiles (Fig. 1A–D). Neuronal groups were identified based on axon trajectories and soma location as established by intracellular recording and labeling in adult goldfish and zebrafish (8).

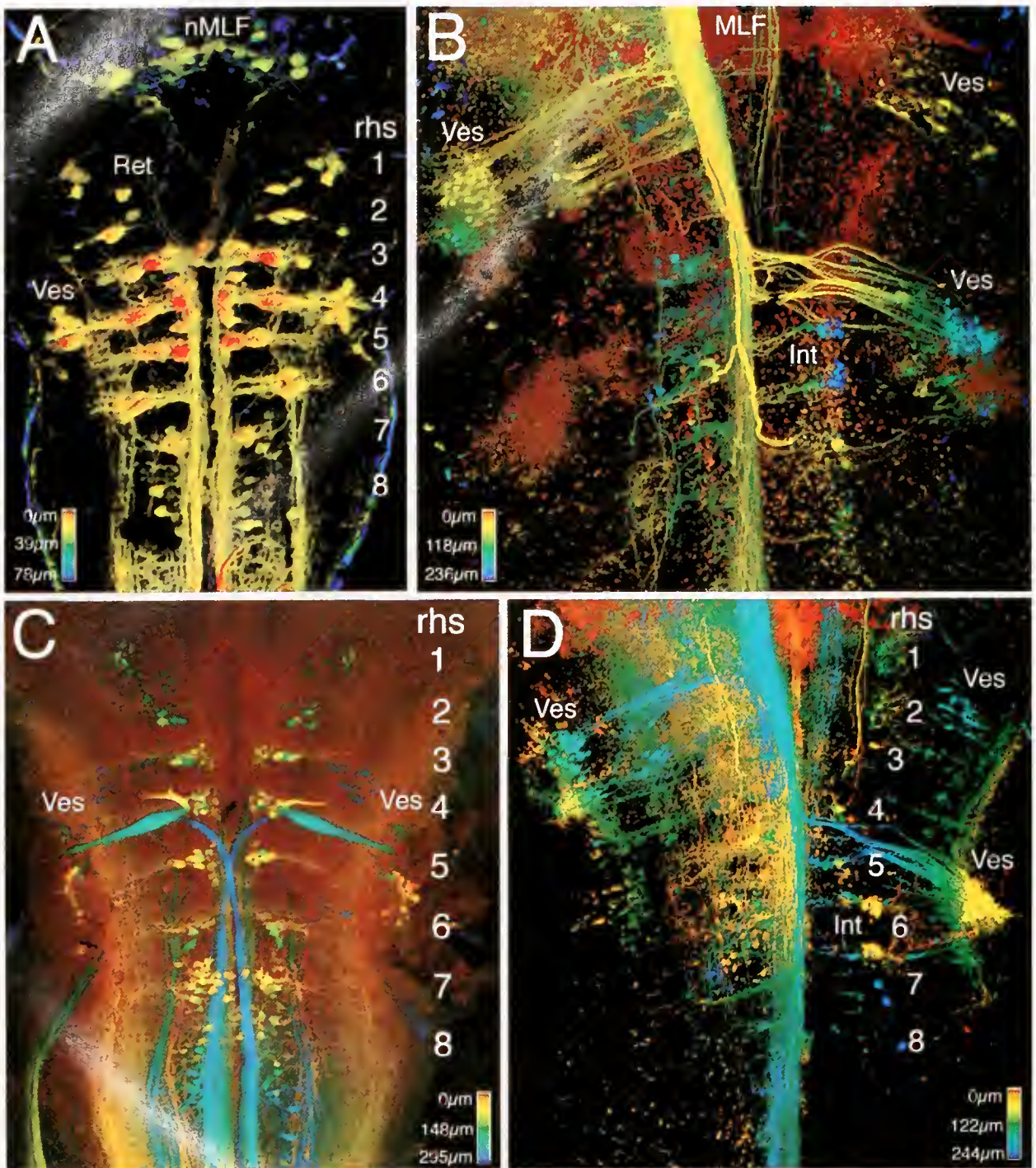


Figure 1. Pseudocolor confocal reconstructions of goldfish and zebrafish hindbrains. Composite profiles were obtained from 40–70 sections in which color indicates depth in microns. Red represents dorsal in A and B and ventral in C and D. Rhombomeric segments (rhs) are numbered 1–8. (A) A 15-day zebrafish larva in which rhodamine was placed on the spinal cord at the level of C2. Eight subsets of reticular neurons (rhs 1–8), three subgroups of vestibular neurons (rhs 3–6), the Mauthner cells (rhs 4), and nMLF neurons were labeled. (B) Unilateral selective label of the left MLF in a 50-day goldfish hindbrain showing ipsilateral (rhs 2–4) and contralateral vestibular (rhs 1–2 and 4–6) neurons. Internuclear neurons were located in rhs 5 and 6. (C) A 55-day goldfish hindbrain labeled from spinal cord at C2. The segmented reticular vestibular scaffold was similar to that described in the zebrafish. (D) Unilateral midbrain application in 50-day goldfish. Location of vestibular and internuclear neurons was the same as described in C; however, more reticular neurons were labeled in the hindbrain (rhs 1–6). Abbreviations. Int, internuclear; MLF, medial longitudinal fasciculus; nMLF, nucleus of the MLF; rhs, rhombomeric segment; Ves, vestibular neurons. Ret, reticular neurons.

Reticular neurons were arrayed within a rhombomeric scaffold of eight segments in both species as illustrated in Figure 1A, C. Reticular neurons projected predominantly to the spinal cord (Fig. 1A, C), but a few neurons in each segment were labeled by mid-brain dye applications (Fig. 1D). Notable among the ascending neurons were the group of "T reticular interneurons" (4), including a pair of "giant fiber" neurons (9), located in rhs 7 and 8 (Fig. 1B). Most of the ascending reticular neurons probably target midbrain premotor nuclei, rather than oculomotor nuclei, because intracellular dye injections of axons projecting to the oculomotor nuclei labeled very few reticular neurons (8).

The vestibular neurons were also largely segregated with respect to spinal and oculomotor targets (compare Fig. 1C with 1B, D). In agreement with the present results, intracellular analysis of vestibular neurons demonstrates that only those from the tangential complex in rhs 4–5 project in common to both oculomotor and spinal targets (8). The segmental relationships of the vestibular neurons were not as clearly definable as those of the reticular neurons (Fig. 1C, D). For example, the ipsilateral projections in the ascending pathways spanned rhs 1–3, and the neurons with contralateral axons were located in rhs 1–2 and 4–6 (Fig. 1B, D). Likewise, the descending vestibular projection originated across rhs 3–6 (Fig. 1A, B).

In summary, comparison of ascending and descending hind-brain projections in larval and juvenile fish leads to two conclusions. First, the premotor reticular and vestibular neurons

responsible for postural control of the body and eyes are highly segregated with respect to both segmental location and targets. Second, only a very small subset of vestibular neurons, and possibly no reticular neurons, innervate both oculomotor and spinal motor nuclei. These observations also suggest that the segmented adult neuronal phenotype results directly from the retention of an embryonic rhombomeric blueprint.

This work was supported by NIH grants EY-02007 and RR-10291.

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Reference: *Biol. Bull.* 191: 259–260. (October, 1996)

The Temporal Transfer Function of the *Limulus* Lateral Eye

E. Kim¹, C. Passaglia², F. Dodge³, and R. B. Barlow³ (Marine Biological Laboratory)

Extensive studies of the lateral eye of the horseshoe crab, *Limulus polyphemus*, have revealed important facets of visual physiology (1). Among these are the excitatory and inhibitory neural mechanisms that shape retinal responses to both spatial and temporal changes in light intensity.

Two studies of the temporal response properties of the eye (2, 3) show that the retina *in situ* is more sensitive to light flickering at higher temporal frequencies than previously reported for excised eyes (4), but the studies differ in their frequency of peak sensitivity. In brief, the temporal transfer function (TTF) measured by Brodie *et al.* (Ref. 2, solid line Fig. 1A) exhibits peak sensitivity in the range of 5–6 Hz with a gain of 10, whereas that measured by Batra and Barlow (Ref. 3, dashed line in Fig. 1A) peaks in the range of 3–4 Hz with a gain of only 5. Both studies measured TTFs of single ommatidia with light modulated by a sum-of-sinusoids technique (5). The former study, however, illuminated the entire retina, whereas the latter study limited the stimulus to a single ommatidium. Thus, the difference between the TTFs in Figure 1A (solid vs. dashed curves) may result from lateral inhibitory influences.

In our initial studies of the temporal properties of the eye, we found that the average TTF measured without background

illumination ($n = 5$, Florida and Woods Hole horseshoe crabs) matched that measured by Batra and Barlow (3). In contrast, we found that the average TTF with background ($n = 3$, Florida horseshoe crabs) matched that measured by Brodie *et al.* (2). These results suggest that constant inhibition from surrounding receptors changed the temporal response properties of the recorded ommatidium. Note that all of these experiments were performed with different animals. If the change in TTFs in Figure 1A results from lateral inhibition, then we should be able to shift from one TTF to the other by turning the background illumination on and off in the same animal.

To carry out this experiment, we cut a 20-cm hole in the carapace anterior to one lateral eye and slipped a chamber with a built-in microsuction electrode around the optic nerve trunk. We teased away a nerve fiber of an ommatidium located at the center of the eye and pulled it into the microsuction electrode to record its response (6). We submerged the animal, which was stabilized on a platform, in a seawater tank containing a transparent glass window. We then aligned the optic axis of the recorded ommatidium with the center of a display monitor (Model 608 Tektronics) located on the other side of the glass window at a distance of 4.5 cm from the lateral eye containing the recorded ommatidium. We measured the TTF of the single ommatidium using a small spot (6° visual angle) modulated by a sum of sinusoids (Ref 5, Model 1010 Venus Visual Stimulator, Neuroscientific Corp.), with and without background illumination.

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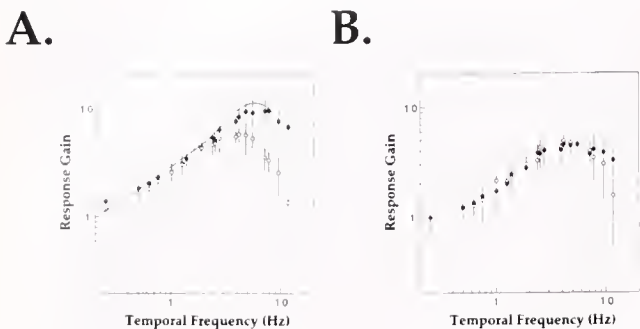


Figure 1. (A) Temporal transfer functions measured by Batra and Barlow (dashed line), Brodie et al. (solid line), and in our preliminary experiments with (filled circles) and without (unfilled circles) background illumination. (B) TTF from three single ommatidia in as many eyes. Filled circles denote responses recorded with constant background illumination and unfilled circles those recorded without background. Error bars denote standard deviation.

Figure 1B shows the average TTF measured with (filled circles) and without (unfilled circles) background illumination from three ommatidia in as many eyes. Note that background illumination did not change the shape of the TTFs, indicating that constant lateral inhibitory inputs do not influence the temporal response properties of an ommatidia. However, past studies show that modulated lateral inhibitory inputs generated

by flickering backgrounds can indeed change temporal response properties (6). The close correspondence of the TTFs measured with and without background in Fig. 1B strongly suggests that the different shaped TTFs in Fig. 1A do not result from the effects of lateral inhibition. The cause of the differences is not known but may result from differences among horseshoe crabs. We are also exploring the possibility that the ambient temperature of a crab's seawater environment before surgery may influence temporal response properties of its eyes.

Supported by grants from the National Science Foundation and the National Institute of Health.

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Lead Affects Learning by *Hermisenda crassicornis*

Alan M. Kuzirian (Marine Biological Laboratory), Frank M. Child, Herman T. Epstein, Peter J. S. Smith, and Catherine T. Tamse¹

Research on lead has a long scientific history, the metal having first been identified as a neurotoxin over 200 years ago. In humans, average blood levels of lead have dropped since 1978, from over 20 to less than 3 $\mu\text{g}/\text{dl}$ (30 ppb) (1). Because lead has multiple sites of action, the mechanisms underlying its toxicity are complex, and its effects on human health and development are multifarious, ranging from alterations in intracellular metabolic pathways and heme synthesis to lowered intelligence scores to increased aggressive behavior (2).

Blood levels of lead above 10 $\mu\text{g}/\text{dl}$ cause demonstrable intelligence or behavioral deficits in children (3). Because many physiological properties underlying learning and memory are known for the nudibranch mollusc *Hermisenda*, we have been investigating whether this animal can effectively serve as a model for lead toxicity studies (4,5). In this paper, we report the first effects of lead on learning in *Hermisenda*.

Upon arrival from Sea Life Supply (Sand City, CA), the animals were adapted to lab conditions for three days. They were initially tested and grouped for similar robust behavioral responses. Subsequently the nudibranchs were placed in either natural seawater (NSW; control animals) or NSW containing 4.76 mg/l lead acetate (experimental animals); all animals were fed a daily diet of the cnidarian hydroid *Tubularia sp.* After three undisturbed days of lead exposure, all animals underwent three days of associative (Pavlov-

ian) conditioning (6), consisting of 50 trials of light (condition stimulus; CS) paired with agitation (unconditioned stimulus; UCS). The animals were tested the following day for evidence of behavioral conditioning by measuring foot contraction in response to light alone (7). The animals' responses were scored as follows: contraction, no-response, or foot extension.

In all three experiments reported here, lead significantly reduced the ability of *Hermisenda* to undergo associative conditioning (Fig. 1A–C). Of the animals in the three control groups ($n = 56$), 40–70% (12/32, 11/17, 5/7 respectively) exhibited the positive foot contraction response (Fig. 1B), while an average of only 9.3% (4/43; $n = 43$) of those exposed to lead contracted (Fig. 1C). Most animals exposed to lead either did not respond to the condition stimulus (28/43), or their foot actually extended in normal locomotory behavior (11/43; Fig. 1A). The combined experimental data were subjected to Chi-Square analysis for binned data between lead and no-lead, yielding a Chi-Square value of 20.190 ($df = 2$), with a probability of <0.0001 (Fig. 1C). Clearly, lead significantly affected the ability of *Hermisenda* to acquire the associative learning, behavioral change of foot contraction elicited by the CS, light.

The lead exposure levels used in these experiments were higher than those acceptable for human circulating blood levels (1,3). However, they are within the range typically used to challenge and raise vertebrate lead levels, and equal to those used in other studies on neurologic effects in invertebrates (8,2).

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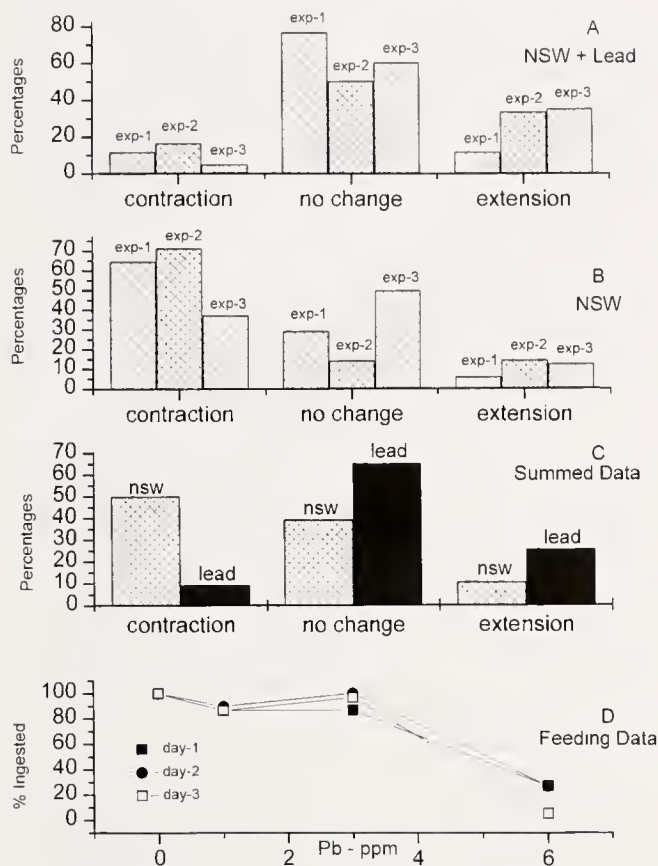


Figure 1. Summary data of lead effects on *Hermissenda* associative learning and feeding: **A.** Graph of three experiments of *Hermissenda* exposed to lead (4.76 ppm), trained and tested for learning response: foot contraction, positive learning acquisition; no response or foot extension, no learning ($n = 43$). **B.** Graph of responses of control animals kept in natural seawater (NSW), trained and tested as in **A** ($n = 56$). **C.** Graph of summed experimental data. A Chi-Square value of 20.19 with a probability of <0.0001 was obtained between lead and no-lead ($df = 2$). **D.** Graph of the percentage of *Tubularia* polyps consumed over a three-day period by *Hermissenda* exposed to 0, 1, 3, and 6 ppm lead ($n = 9/\text{group}$) illustrating a marked feeding inhibition above 3 ppm.

Ancillary to this study we found that feeding in *Hermissenda* exposed to lead above 3 ppm is depressed (94% of *Tubularia* polyps consumed at 0,1,3 ppm, $n = 27$; 19.4% of polyps consumed at 6 ppm, $n = 9$; Fig. 1D). Feeding is also regulated by serotonin and can

be similarly depressed by its toxic analog, 5,7-dihydroxytryptamine (5,7-DHT) (12). Lead interferes with neurotransmitter synthesis, serotonin (5-HT) and GABA in particular (14, 15). 5-HT is known to modulate long-term memory in *Aplysia* (9) and, with GABA, conditioning responses in *Hermissenda* (10,11). Therefore an approach to studying the neurotoxic effects of lead on *Hermissenda* learning might be through the synthesis of neurohormones like serotonin and GABA and their neuromodulatory pathways. These studies would be enhanced if coupled with pharmacological experiments using therapeutic lead chelators such as sodium 2,3-dimercaptopropane-1-sulphonate (DMPS) and meso-2,3-dimercaptosuccinic acid (DMSA) (13). *Hermissenda* appears to have excellent potential for use as a non-mammalian model for lead toxicity.

This research was funded in part by an NCRR-NIH grant (P40-RR03820) to A.M.K. and by funds from the MBL to A.M.K. and P.J.S.S. Technical and equipment support was also given by B.G. Schreurs and D.L. Alkon and is gratefully acknowledged.

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Ion Fluxes from Skate Retinal Horizontal Cells Measured Using Self-Referencing Ion-Selective Electrodes

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The establishment and maintenance of ionic gradients across cellular membranes is of fundamental importance for cells in establishing normal patterns of communication. A wide variety of channels and transport proteins act in a coor-

dated fashion to permit a differential distribution of relevant ions. Voltage-clamp studies have provided much useful information about the contributions that electrogenic transporters make to the establishment of such gradients. However, many electrically silent processes also contribute to the total ionic flux across the membranes of cells, and these are not readily examined with voltage clamp methods. The ac-

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tivity of such transporters can be monitored with radioactive tracers in flux experiments, but the self-referencing ion-selective probes used in the following study offer far greater time and spatial resolution.

Ion-selective electrodes have long been used in studies of the intracellular and extracellular distribution of different ion species [see Ammann (1)]. In a variation of this technique, a self-referencing microelectrode with greatly enhanced sensitivity and stability is used and permits examination of the small ionic gradients generated at the plasma membrane-saline interface [(2); see Smith, Sanger, and Jaffe (3) for review]. In brief, a single ion-selective electrode is moved alternately between a point less than 1 μm from the cell membrane and one 10 μm further away in the surrounding medium. The voltage difference between the readings at the two positions reflects the difference in the free ion concentrations at those positions. The readings are extensively averaged, improving the signal-to-noise ratio. The rate of probe movement (≈ 0.3 Hz) is a compromise between the time required for the ionophore to respond to the new local ion concentration and the speed required to minimize signal drift (2). Measurements are taken close to the plasma membrane, as the gradient drops off rapidly. Control measurements are taken at least 40 μm distant from the cell surface. In no case could a differential voltage measurement be detected in a control position.

The purpose of the present study was to determine whether such self-referencing, ion-selective electrodes could be used to detect steady ion fluxes from retinal horizontal cells of the skate. In these relatively large cells one can examine a variety of ion transport mechanisms, including ion flux resulting from sodium-potassium ATPase activity (5) and the sodium- and chloride-dependent transport of the neutral amino acid GABA (6). The present experiments were conducted on isolated external horizontal cells obtained by dissociating retinas with papain (7).

Ion-selective electrodes were prepared as follows: thin-wall glass capillaries (o.d. 1.5 mm) were pulled to tip diameters of ≈ 2 μm , silanized, and backfilled with an electrolyte (100 mM calcium chloride for calcium-selective electrodes, 100 mM potassium chloride for proton-selective electrodes). The electrolyte was forced to the tip by applying air pressure to the back of the pipette, following which the tip was rapidly placed in contact with the appropriate ion-selective cocktail. When the pressure was relieved, the ion-selective compound was drawn into the pipette; the length of the column could be controlled, and was typically about 30 μm .

The following commercially available ion-selective cocktails were used in making the electrodes: Calcium Ionophore 1—Cocktail A, Fluka Corp; Hydrogen Ionophore 1—Cocktail B, Fluka Corp; Potassium Ionophore 1—Cocktail B, Fluka Corp. The calcium ionophore cocktail is highly selective for calcium over other cation species, being approximately 5 log units more sensitive to calcium than to sodium, potassium, or magnesium; the hydrogen ion cocktail possesses an even greater selectivity, being almost 10 log units more sensitive to protons than to potassium and sodium (5). No flux is recorded in steady-state using a potassium ionophore probe. In our application there is virtually no possibility of sodium interference due to the high sodium background, selectivity coefficients, and the Nernstian characteristics of the cocktails employed. To calibrate the electrodes and ascertain that their response was indeed Nernstian, readings from calcium-selective electrodes were obtained in solutions containing 10, 1, and 0.1 mM calcium prepared in distilled water; standard pH buffer solutions

ranging in pH from 6.0 to 8.0 were employed in calibrating the proton-selective electrodes.

Investigations of calcium flux from the retinal horizontal cells were conducted in a saline whose external calcium was reduced from 4 mM to 100 μM , greatly improving the signal-to-noise ratio. No calcium buffer was present. Studies with proton-selective electrodes were conducted in a saline containing 2 mM HEPES in place of the normally applied 10 mM HEPES.

Horizontal cells displayed an apparent steady-state efflux of calcium under these experimental conditions. A differential voltage of -48.5 ± 14.5 μV was detected from nine cells. The measured voltage difference ΔV detected with the probe can be converted to a concentration difference ΔC using the equation: $\Delta C = 2.3 (\Delta V/C_b/S)$, where S is the Nernstian slope of the electrode in mV, and C_b is the background concentration of calcium (3). The concentration difference can be converted into an ion flux (J) by using the equation: $J = -D (\Delta C/\Delta r)$, where D is the diffusion constant for calcium and Δr is the distance between the two electrode positions (3). In these experiments with a background calcium concentration near 100 μM , we find that the horizontal cells have a steady-state efflux of 3.75 ± 1.5 $\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$. Use of the hydrogen-selective probe revealed a voltage differential of 31 ± 11 μV , corresponding to an efflux of protons from the cells. Conversion of μV values to flux is complicated for hydrogen ions by the presence of a buffer in the medium, a problem considered in detail by Demarest and Morgan (8).

These results demonstrate that ion-selective, self-referencing probes may indeed be useful in studying ion fluxes across retinal cells. Future studies, combining the ion-selective probes with whole-cell clamping methodology and cytosolic free-ion imaging, promise to provide new information about the mechanisms responsible for maintaining ionic gradients across the membranes of these retinal neurons, and about the function of these ionic movements.

We would like to thank Richard H. Sanger for unsurpassed electronic and computer assistance, Kasia Hammar for expert assistance with cell culture and preparation of solutions, and Rudy Rottenfusser of the Zeiss Corporation for generous help with microscopy and loan of microscopic equipment. This work was supported by grants P41 RR01395 from the National Center for Research Resources to PJSS and EY09411 from the National Eye Institute to RPM.

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Protein Synthesis in the Presynaptic Endings of the Squid Photoreceptor Neuron: *In vitro* and *in vivo* Modulation

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In previous experiments we have shown that the synaptosomal fraction from squid optic lobe incorporates [³⁵S]-methionine into protein in a reaction that is largely inhibited by cycloheximide, sensitive to the ionic composition of the medium, completely unaffected by RNase, and fully inhibited by a hypo-osmotic treatment (1). These features indicate that the active particles are surrounded by a plasma membrane enclosing eukaryotic polysomes and the soluble factors required for protein synthesis. The synaptosomal synthetic activity was attributed to the large nerve terminals enriching the fraction, as the synaptosomal translation products (including immunoabsorbed neurofilament proteins) were found to markedly differ from the proteins synthesized by nerve cell bodies or glial cells (1–3). More recently, this conclusion was confirmed by light and EM autoradiographic analyses of synaptosomal samples incubated with [³H]leucine which showed most silver grains to be consistently overlying the large presynaptic terminals (4). In addition, using the method of electron spectroscopic imaging (5), ribosomes and polysomes were clearly identified (i) within the same type of nerve endings in the synaptosomal fraction (4), and (ii) in the presynaptic endings of retinal photoreceptor neurons that terminate in the cortical layers of the optic lobe (Martin *et al.*, unpub. data). Because these are the only large nerve terminals present in the optic lobe (6), we can identify the photoreceptor neurons as the source of the large nerve terminals of the synaptosomal fraction.

Our previous observations that synthesis of synaptosomal protein is strongly inhibited by calcium ionophores and other compounds known to increase the intracellular levels of Ca⁺⁺ ions and, conversely, by specific inhibitors of calmodulin and calcium-dependent protein kinase (1,3) suggested that the changes in presynaptic levels of Ca⁺⁺ triggered by the activation of the retinal photoreceptors might play an important role in the regulation of presynaptic protein synthesis.

The prevalent contribution of photoreceptor presynaptic terminals to the synthesis of protein in the synaptosomal fraction prompted an investigation of the effect of light stimulation on this activity. In our experiments, one or two live squid were kept for 6–7 hours in a tank with circulating seawater (18–20°C) placed in a lighted room, while one or two control squid were kept in a nearby tank fully covered by four layers of black plastic bags. The rate of protein synthesis by the synaptosomal fractions prepared from the optic lobe was determined according to routine methods (1). In eight experimental pairs, the specific activity of the protein from light-adapted animals (915 ± 304 cpm/μg protein: average value with SD) was significantly higher ($P < 0.02$; *t*-test for unpaired data) than that of squid kept in the dark environment (542 ± 190 cpm/mg protein). As the difference was found between matched animals at different times of the day (morning or evening hours), the data cannot be attributed to circadian influences, but rather indicate a light-induced effect, presumably mediated by activation of the retinal photoreceptors.

Taken together, our observations indicate that the large nerve endings of the squid photoreceptor neuron contain a system of eukaryotic polysomes whose activity is modulated by exposure of the squid to a light or dark environment.

We acknowledge the financial support of USPHS grant NS30715, of EC grant C11*-CT93-0037, and of grants provided by CNR (PS Mezzogiorno), MURST, and the University of Naples, Italy.

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***Limulus* Retinal mRNA Induces Light-Dependent Currents in *Xenopus* Oocytes**
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*B. E. Knox*², and *R. B. Barlow, Jr.*²

We are investigating the expression of *Limulus* retinal mRNA in *Xenopus laevis* oocytes as a means for examining the properties of *Limulus* rhodopsin that may influence photoreceptor sensitivity and noise (1,2). *Xenopus* oocytes were chosen because they contain G-protein-mediated ionic conductances (3) and can express mRNA from both vertebrate and invertebrate retinas (4,5). We report here that oocytes injected with *Limulus* retinal poly A⁺ RNA and incubated with 11-cis retinal exhibit light-dependent ionic currents. This finding demonstrates that *Limulus* retinal mRNA is efficiently translated by *Xenopus* oocytes and is able to direct the synthesis of proteins necessary for light transduction.

RNA from retinas excised from *Limulus* lateral eyes was purified using an acid guanidium method (6), and poly A⁺ RNA was selected using immobilized oligo dT (Promega Company). The RNA was ethanol-precipitated twice, quantitated by UV spectroscopy, and resuspended in water for microinjection.

To obtain oocytes, we anesthetized *Xenopus* frogs with 0.15% tricaine and surgically removed the ovarian lobes. Oocytes were dissociated from the surrounding epithelium and defolliculated using 2 mg/ml collagenase (type 1A Sigma). Stage V and VI cells were microinjected with 50–100 nl of *Limulus* retinal poly A⁺ RNA (0.5 µg/µl in water), and incubated for 2–6 days at 17°C in Barth's solution containing sodium pyruvate (5 mM) and gentamicin (10 µg/ml). We localized injections to the animal pole of each oocyte.

For electrophysiological recording, we placed single oocytes in a recording chamber that was constantly perfused with recording solution and impaled them with two glass microelectrodes (2–4 MOhms). If oocytes had stable resting potentials after about 30 min in darkness, we perfused them with 20 µM 11-cis retinal for 45 min and tested them for light sensitivity. Light from an unfiltered tungsten filament lamp was delivered to the oocyte with a fiber-optic light pipe (0.02 mW/cm² at the surface of the oocyte). Light responses after 3 days of incubation were small or non-existent, but after 4 days they were robust and readily recordable. Five of 10 injected oocytes responded to the light.

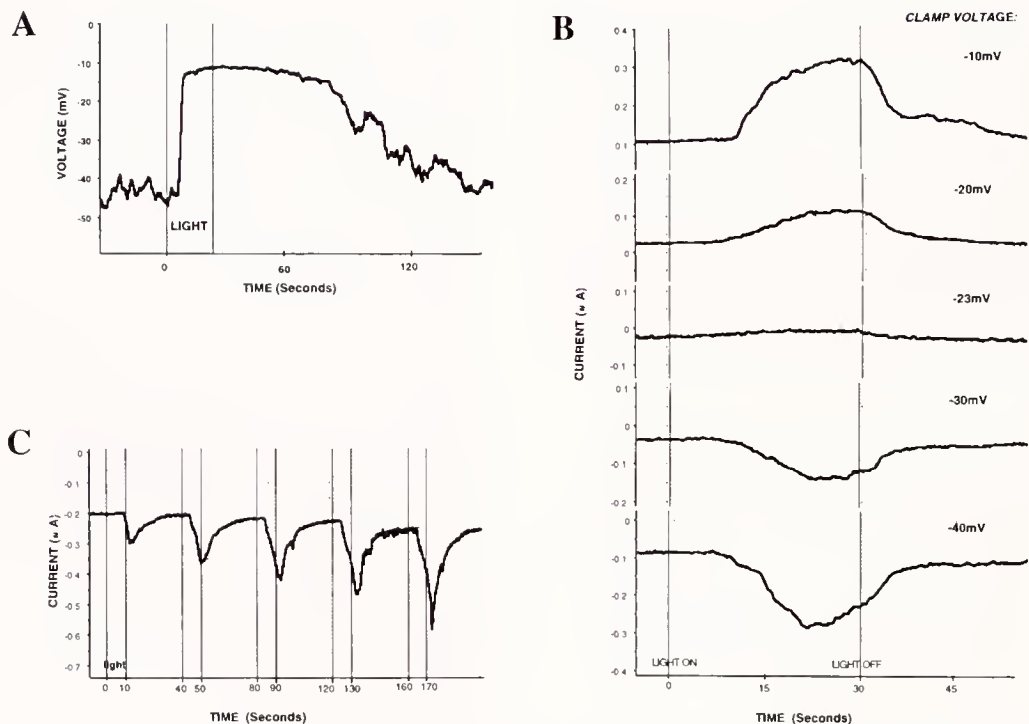


Figure 1. Light-induced current responses from *Xenopus* oocytes injected with *Limulus* retinal poly A⁺ RNA. (A) The response of a single oocyte under current clamp. Oocytes depolarized to between -12 and -24 mV in response to a light flash. (B) The reversal potential of the light-dependent current of a single oocyte under voltage clamp. Currents reversed, on average, at -18 mV. (C) The repetitive responses of an oocyte to repetitive light stimulation. Oocytes maintained light sensitivity without the need for additional incubation with 11-cis retinal.

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Figure 1A shows the current clamp response of an oocyte to a 25-s light flash. After a 6-s delay from light onset, the membrane depolarized to a level of -12 mV, and it returned to pre-stimulus baseline about 1 min after light offset. Responses of all other oocytes were similar to this, depolarizing to -12 to -23 mV, with latencies that ranged from 6 to 24 s. Figure 1B shows the response of another oocyte to light while voltage clamped at various potentials. Light flashes evoked sustained inward currents at clamp potentials more negative than -25 mV, and outward currents at potentials more positive than -20 mV. The cause of the reduction in inward current before light offset at clamp potentials of -30 and -40 mV is not known and was not observed in other oocytes. The reversal potential for the current for this oocyte was -23 mV. Light-evoked currents for other oocytes reversed direction at holding potentials between -12 and -23 mV, suggesting that this response is mediated by the endogenous calcium-activated chloride conductance (7, 8). Many other expressed receptor proteins also are known to couple into this pathway, and the long response latency we observed is typical of the activation of this chloride conductance (9). No light responses were detected in mRNA-injected oocytes before the application of 11-cis retinal. Other studies using the same expression system detected no responses in non-injected oocytes after incubation with 11-cis retinal (5).

Figure 1C shows that repetitive flashes of light can evoke repetitive responses from oocytes without the need for additional incubation with 11-cis retinal. This sustained light sensitivity supports an earlier finding that *Limulus* metarhodopsin is a relatively stable and photoreversible photoproduct of *Limulus* rhodopsin (10). This is not the case for bovine rhodopsin expressed in *Xenopus* oocytes (5), which requires additional incubation with 11-cis retinal to maintain light sensitivity.

Note that the light-evoked currents in Figure 1C increased in amplitude in response to repeated light flashes of a constant intensity, while the response latencies decreased from 10 s for the first response to about 4 s for subsequent responses. Decreasing the intensity of the light flashes decreased both the response amplitude and the steady state level. Flashes of low in-

tensity often failed to evoke any response to the first test flash, but not to subsequent ones (data not shown). Assuming that repetitive flashes of equal intensity generate equal levels of activated rhodopsin, the increasing response amplitude in Figure 1C may reflect the accumulation of an internal transmitter involved in the transduction cascade that yields the light-dependent currents we record. The occasional failure of the first test flash in a series to evoke a response points to the existence of a threshold for action for one or more internal constituents of this transduction pathway. We have not explored this facilitatory response to subsequent light flashes in sufficient detail to determine either the exact threshold for action for the internal transmitter or the time course of its delay.

In conclusion, we have demonstrated that *Xenopus* oocytes efficiently translate *Limulus* retinal mRNA and provide a suitable system for studying the characteristics of light transduction. We will combine this technology with molecular biological techniques to study how the properties of *Limulus* rhodopsin influence photoreceptor sensitivity and noise.

Supported by grants from the National Institutes of Health, and the National Science Foundation.

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Reference: *Biol. Bull.* 191: 265–267. (October, 1996)

Ionophore-Induced Calcium Waves Activate Unfertilized Zebrafish (*Danio rerio*) Eggs

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Egg activation is accompanied by propagating Ca^{2+} waves that can be induced by factors such as sperm, pricking, calcium ionophores, and injected IP_3 or cADPR (1). Such waves have been reported from a variety of deuterostomes and protozoans (2, 3). One notable omission from this ever-expanding

list was the zebrafish (*Danio rerio*). In light of the increasing popularity of this fish as a model for study of vertebrate development, we present data showing that zebrafish eggs are likewise activated by a regenerative Ca^{2+} wave.

In the past, the major obstacle to such an investigation lay in the inability to delay *in vitro* fertilization long enough to load an unactivated egg with a calcium reporter. This difficulty has recently been overcome by the discovery that unactivated zebrafish eggs can be held in Coho salmon (*Onchorhynchus kisutch*) ovarian fluid (Sea Tech Bioproducts) for as long as 1.5 hours and still be successfully activated (4).

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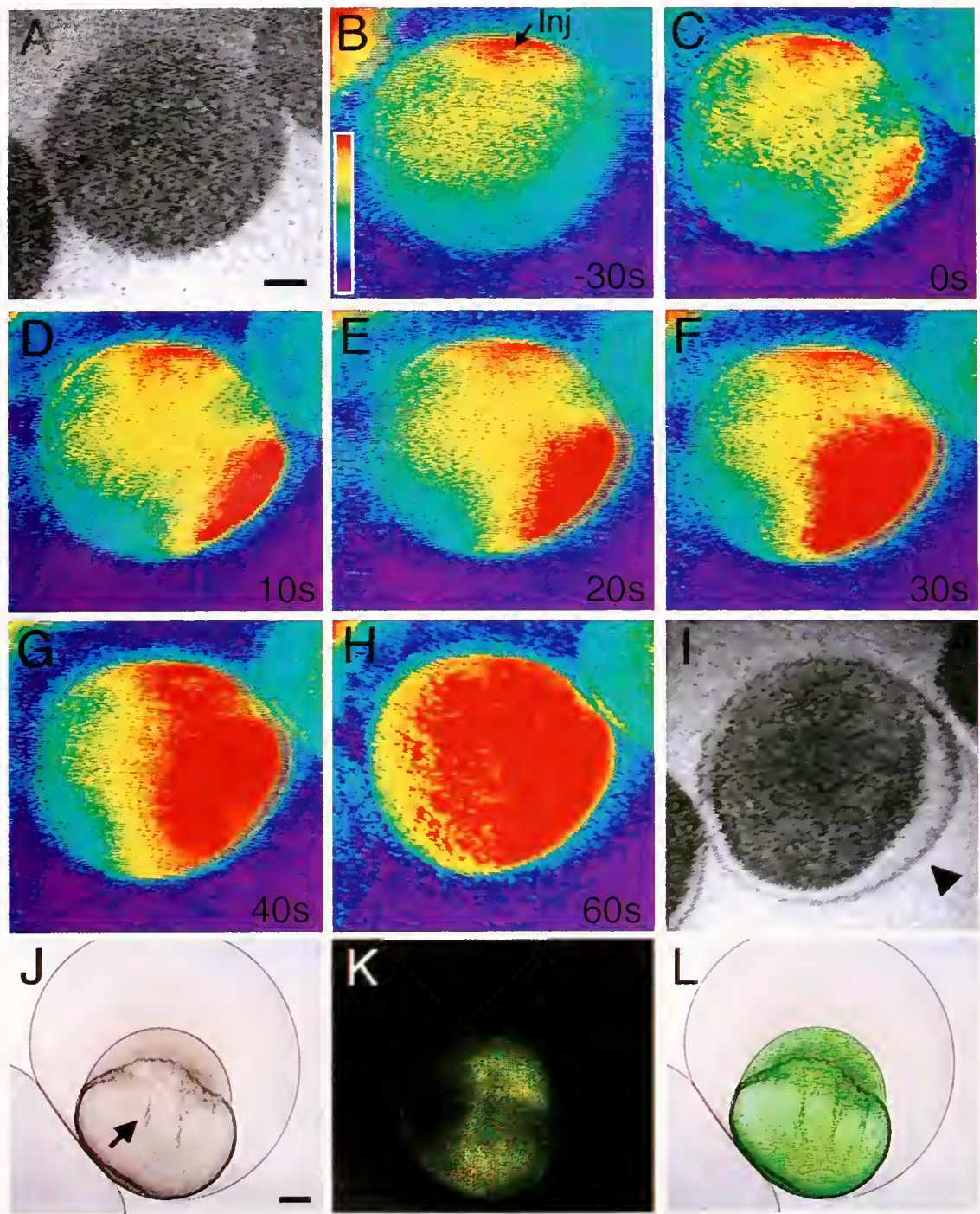


Figure 1. An example of an ionophore-induced Ca^{2+} wave crossing a zebrafish egg. Panel A illustrates a transmitted light image of an unactivated egg. Panels B through H are successive (microchannel-plate-generated) fluorescent images showing a Ca^{2+} wave crossing the egg at a velocity of about $10 \mu\text{m/s}$ (ionomycin solution added at Panel B, inj, indicates the injection site). Panel I is a second transmitted light image collected after the passage of the wave, clearly illustrating that the egg had been activated (arrowhead indicates the rising chorion). Panels J through L show that activated (but unfertilized) eggs loaded with Fluo-3 undergo normal ooplasmic segregation, forming a regular blastodisc (L is an overlay of J and K). It is clear that indicator dye injected into the center of an unactivated egg is carried via ooplasmic streaming (indicated by arrow in Panel J) into the forming blastodisc. Panel J (60 min post-activation) illustrates a fully elevated chorion. Scale bars in Panels A and J equal $100 \mu\text{m}$.

We stripped unactivated eggs from ripe, anesthetized females and immediately immersed them in 100 μ l of salmon ovarian fluid (SOF) in agarose-coated viewing chambers. Eggs were injected with 3 to 5 nl of the calcium reporter Fluo-3 (pipette concentration: 300 μ M) and left for 20 to 30 min to allow the reporter to diffuse throughout the egg. Eggs were then checked for dye distribution by using a multichannel plate detector (Motion Analysis Inc.) mounted on a Zeiss IM35 platform. During each experiment, several eggs were simultaneously imaged through a Zeiss Plan 6.3 $\times$ objective while under the appropriate excitation illumination for Fluo-3. Eggs were checked for signs of activation under brightfield illumination. Both brightfield and fluorescent images were stored on videotape for subsequent analysis.

Eggs were activated by adding 10 μ l of 50 μ M ionomycin. Three distinct observations were made. (1) In cases where there were no signs of activation (46% of the eggs observed), no increases in intracellular Ca^{2+} were detected. (2) Where activation was complete, judged by a comprehensive raising of the chorion (23% of the eggs observed), an accompanying wave of

elevated Ca^{2+} traversed the egg. (3) When we recorded a localized, non-propagating rise in intracellular Ca^{2+} (31% of the eggs observed), it was coincident with localized egg activation, judged by localized raising of the chorion.

Figure 1 shows a representative example of an ionophore-induced Ca^{2+} wave. Such waves took around 60 s to cross the 625- μ m-diameter egg (at 24°C), indicating a velocity of around 10 μ m/s. Thus for zebrafish eggs, our results are consistent with the general observation that an explosive rise in free calcium provides most of the activating stimulus.

This work was supported by NIH R24 RR101291-01A1 awarded to ALM and RTH, and Research Grants Council (Hong Kong) HKUST650/96M awarded to ALM.

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Effect of SR Calcium Pump Inhibition on Relaxation and Power from Scup Red Muscle

Douglas M. Swank and Lawrence C. Rome (University of Pennsylvania)

During cyclical locomotory movements such as fish swimming, muscles alternately become active and relax, shorten and lengthen. For a given muscle to generate large net mechanical power as it shortens, the contralateral muscle (which is being lengthened) must be relaxed, so as not to offer resistance. Hence relaxation rate is an important determinant of muscle power generation (1, 2). Muscles can be built with a very wide range of relaxation rates (3), and this rate is set largely by the density of SR Ca^{2+} pumps. To better understand how mechanical power output is influenced by a muscle's ability to relax, we altered the relaxation rate of isolated red muscle bundles from the scup by using cyclopiazonic acid (CPA) to inhibit their SR Ca^{2+} pumps. Isometric tetanic and twitch relaxation times were measured as tension fell from 90% to 10% of maximum. The effect of a change in relaxation rate on power production was then measured as the muscle worked cyclically (work loop technique (4, 5)). Data are reported as means $\pm$ S.E., $N = 5$ –6.

CPA at 0.5 μ M was the minimum dose that caused a measurable increase in isometric relaxation time, and this effect increased with CPA concentration until the effect saturated at about 50 μ M (Fig. 1A). CPA also caused twitches to have higher peak forces, and tetani to have longer maximum force durations. CPA at 1.5 μ M increased peak twitch force 18% and prolonged, by 17.2%, the time that maximum force was maintained during 125 ms tetani; from 62 ± 4.5 ms to 79.2 ± 8.1 ms. These results suggest that, after stimulation ends, CPA prolongs the time [Ca^{2+}] remains above the level that saturates troponin. Increasing stimulation duration, though it mimicked the CPA-induced prolongation of maximum force, did not slow relaxation time, suggesting that CPA also slows the falling phase of the calcium transient.

Slowing the relaxation rate with CPA had a dramatic (and seemingly disproportionate) effect on red muscle power production. For instance, when the relaxation rate was slowed by 64% with 2 μ M CPA, the mechanical power output at 4.7 Hz (the tailbeat frequency used during swimming at 50 cm/s) was reduced 3-fold (Fig. 1B). As might be expected, the decrease in power by a given dose of CPA depends on the oscillation frequency of the muscle. For instance 1.5 μ M CPA, which increased relaxation time from 92.9 ± 11.6 ms to 167.2 ± 20.1 ms, caused 19%, 47%, and 84% decreases in power at 2.5 Hz, 4.7 Hz, and 6.4 Hz (measured under stimulus and length change conditions that maximized work at the given frequency). The decrease in power was primarily due to high force during the lengthening half of the work loop cycle, and thus high negative work values. At an oscillation frequency of 4.7 Hz, 1.5 μ M CPA caused a 3.5-fold increase in negative work, -1.06 ± 0.21 to -3.77 ± 0.63 μ J, which resulted in a net power decrease from 46.8 ± 5.2 W kg^{-1} to 24.9 ± 3.3 W kg^{-1} .

To better assess the seemingly disproportionate effect of CPA on power production, we compared this effect with the natural difference in relaxation and power found between anterior and posterior red muscle in scup (6). The slower posterior relaxation rate could be duplicated by exposing anterior bundles to 1.6 μ M CPA. However, the CPA-modified anterior muscle, though having the same relaxation time from 90% to 10% of tetanic force, produced far less power than a posterior muscle. This was due to higher force during the lengthening phase of the work loop. To duplicate the posterior muscle's lower power production only 0.5 μ M CPA was needed.

Taken together, these results suggest that there is a fundamental difference between altering the relaxation rate by vary-

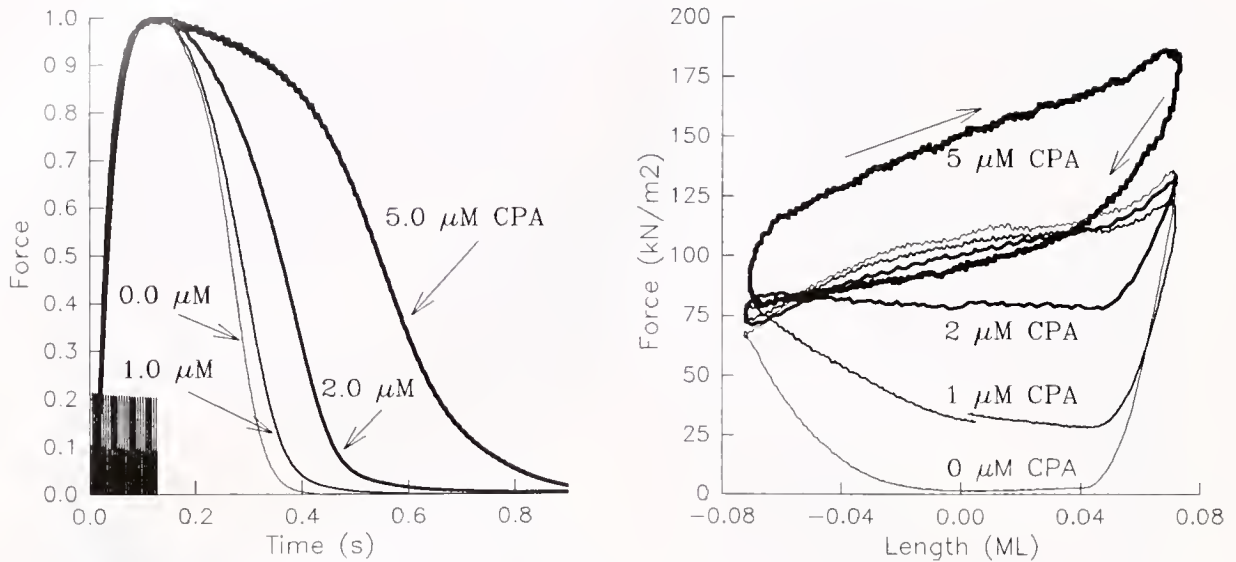


Figure 1. (A) Tetanic force traces after addition of progressively higher concentrations of CPA on a red muscle bundle. Force traces are normalized to the maximum force of each tetanus. Each tetanus had a stimulus duration of 125 ms (shown in bottom left of figure). $T_{1,90-10}$ relaxation time increased from 133 ms prior to CPA to 163, 218, and 395 ms after 1.0, 2.0, and 5.0 μM CPA, respectively. (B) Effect of the different relaxation times shown in (A) on power production. Work loops are at 4.7-Hz muscle oscillation frequency, 7.7% strain, -48° phase, and 75-ms stimulus duration. Power values decreased from 46.8 W kg^{-1} prior to CPA to 34.2 W kg^{-1} , 15.4 W kg^{-1} and, -29.7 W kg^{-1} after 1.0, 2.0, and 5.0 μM CPA. Note that at 5 μM the work loop is generated in a clockwise direction, indicating that the muscle is having net work done on it (negative work). All other work loops are counterclockwise (positive work). Length changes are given in muscle lengths (ML).

ing the number of Ca^{2+} pumps, and pharmacologically slowing the pumps already present. When the relaxation rate of the posterior muscle is mimicked by the addition of CPA to anterior muscle, the tension falls with a nearly identical time course over most of the force range. However, the relaxation rate deviated at the lower end (20% maximal force to baseline), approaching baseline far more slowly than in the unaltered posterior muscle. This effect grew more pronounced with increasing doses of CPA (Fig. 1A). CPA seems to have a greater inhibitory effect on Ca^{2+} pumps at low $[\text{Ca}^{2+}]$, which allows a small population of cross bridges to remain in force-producing states. Although these few remaining cross bridges have an almost imperceptible effect on isometric relaxation, they generate high forces during the lengthening phase of the work loop (Fig. 1B), thus greatly reducing power.

In conclusion, the results demonstrate that relaxation rate is a major determinant of mechanical power production during cyclical length changes: slowing relaxation rate reduces power

output. The quantitative nature of this relationship, however, is mechanism-dependent; it is much larger during pharmacological slowing of relaxation, presumably because of the prolonged attachment of a small population of crossbridges. The results also illustrate that the work loop technique is more sensitive to changes in relaxation than isometric measurements, and should be employed in evaluating pharmacological interventions on *in vivo* muscle performance.

Support by NIH Grant AR38404 and NSF IBN-9514383.

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Digital Unsharp Masking Reveals Fine Detail in Images Obtained with New Spinning-disk Confocal Microscope

Shinya Inoué (Marine Biological Laboratory) and Ted Inoué¹

The confocal microscope scans the specimen point by point with a minute (diffraction-limited) spot of light, and it prevents light originating from all regions other than that minute spot from entering the photodetector; thus it provides a sharp image of the specimen that is not blurred, *e.g.*, by out-of-focus fluorescence (1). The laser point-scanning confocal microscope is especially effective in generating clear, sharp optical sections of fluorescent specimens. However, most point scanning systems require several seconds to capture images that are sufficiently free from photon noise.

In the scanning-disk-type confocal microscopes, multiple pinholes are located on a spinning Nipkow disk. The pinholes simultaneously illuminate many points on the specimen and also filter out the unwanted scattered light originating from regions not illuminated by the focused image of the pinholes. With the scanning-disk type, the image is generated in real time so that the confocal image can be viewed through the eyepiece and the specimen scanned or focused without waiting for the picture to appear on a monitor screen. However, most of these systems were limited by the low throughput of light (*ca.* 1%) through the pinholes on the Nipkow disk, the presence of residual scan lines, and the imperfect removal of haze originating from fluorescent regions far from focus.

A new system, just introduced, incorporates a second Nipkow spinning disk containing some 20,000 microlenses that are aligned with the same number of pinholes in the main Nipkow disk (Yokogawa Electric Co., CSU-10). The microlenses in this unit improve the throughput of the disk by some 50-fold so that fluorescence images are now bright enough to be seen through the eyepiece under normal room lighting conditions. The new

compact unit can be attached to most upright or inverted microscopes and provides stable images with good in-plane as well as z-axis resolution. Its patented pinhole arrangement eliminates all traces of scan lines. The disk in a prototype high-speed confocal unit is also spinning fast enough that, with the unit equipped with an intensified video camera and high-speed video recorder, movies of fluorescent blood cells circulating in exposed vessels have been made by capturing full frame every 4 ms.

We have tested this new confocal system on biological specimens and have added a further improvement to the image by digital unsharp masking. As shown in panels A and B in Figure 1, the new confocal system does provide optical sections of fluorescent objects that are considerably improved over conventional epifluorescence. The sensitivity was high enough to directly visualize and focus 50-nm-diameter fluorescent beads (courtesy of Dr. Stephen Smith). The image still suffers from haze introduced by the out-of-focus fluorescence characteristic of multiple pinhole systems, but the intruding scan lines are totally absent. When we applied a digital unsharp masking algorithm (in MetaMorph, Universal Imaging Corp.), the haze disappeared, and the image became exceptionally crisp as seen in Figure 1C. Unsharp masking (which is simpler and considerably less intensive computationally than digital deconvolution) suppresses the low spatial frequency components in the image and enhances the contrast of the high spatial frequency components, thus giving rise to the type of image improvement demonstrated. Images of the same specimen obtained on a compact point-scanning laser confocal unit (Nikon PCM-2000 with 60/1.40 Plan Apo objective; *ca.* 10-s scan with 488-nm excitation using similar laser power) displayed less haze but comparable resolution to that with the CSU-10 unit before unsharp masking.

¹ Universal Imaging Corp.



Figure 1. Optical section of dandelion pollen grain autofluorescence observed with: (A) standard epifluorescence, (B) Yokogawa confocal, (C) unsharp masking of B. All images were taken on a Princeton Instruments chilled CCD Camera (MicroMax with KAF-1400 chip integrated for 2.3 s with no binning) with a Zeiss 40×/1.3NA Plan Neofluar objective lens and 488-nm excitation (10 mWatt at CSU-10 input) on a Zeiss Axiovert microscope. Diameter of pollen grain = *ca.* 25 μ m.

By combining the real-time (and faster-than-video rate) direct-view capability of the microlens-equipped scanning-disk confocal unit with digital unsharp masking, we believe that biologists now have available a powerful new tool for imaging and analyzing dynamic events in cellular and molecular studies.

We thank T. Akiyama of Yokogawa Electric Co., R. Rottensuss of C. Zeiss Inc., and M. Christianson of Princeton Instru-

ments Co. for loan of equipment, and B. Amos of Cambridge Univ. and M. Terasaki of MBL for providing fluorescent samples. Supported in part by NIH grant R37 GM31617.

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Birefringence Measurements of the Actin Bundle in the Acrosomal Processes of *Limulus* Sperm

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Actin bundles play an important role in many kinds of cell activities, such as muscle contraction, cytoplasmic streaming, cell migration, and acrosomal reaction. Using our modified polarized light microscope (1), we have measured the birefringence of well-characterized actin bundles to estimate the birefringence of a single actin filament.

The actin bundles in *Limulus* sperm contain a nearly constant number of actin filaments (80 filaments in the basal region) (2). The actin bundle is discharged from the head of the sperm after an acrosomal reaction and thus is very easy to identify.

Limulus sperm shows two types of discharge of acrosomal process—true (Fig. 1A) and false (Fig. 1B). True discharge is part of the normal acrosomal reaction, but false discharge is induced by some stress (for example a temperature increase). In both cases, the discharged process consists of an actin bundle and a membrane (2).

To eliminate the effect of cell membrane on the measurements of retardance, we treated the sperm with Triton X-100 (3). Figure 1A shows a Triton-extracted sperm with a true discharge. Birefringence of the actin bundle is larger in the region nearer to the cell body (Fig. 1A). The birefringence, expressed as retardance, was almost constant in the basal region (0.85 ± 0.11 nm, mean $\pm$ SD, $n = 5$). This observation is consistent with previous findings (2) that the number of actin fibers contained in the basal region is almost constant.

Because electron microscopic observations have shown that the number of the actin filaments in the basal region of the true discharge corresponds to the number in the tip region of the false discharge (2), we compared retardance in the base of the true discharge with that in the tip of the false discharge. The retardance of the tip of the false discharge was 0.72 ± 0.038 nm ($n = 3$), which is similar to the retardance measured in the basal region of the true discharge.

To find the retardance of a single actin filament, we divided the measured retardance of the acrosomal process by its number of actin filaments (80). As has been shown previously (4),

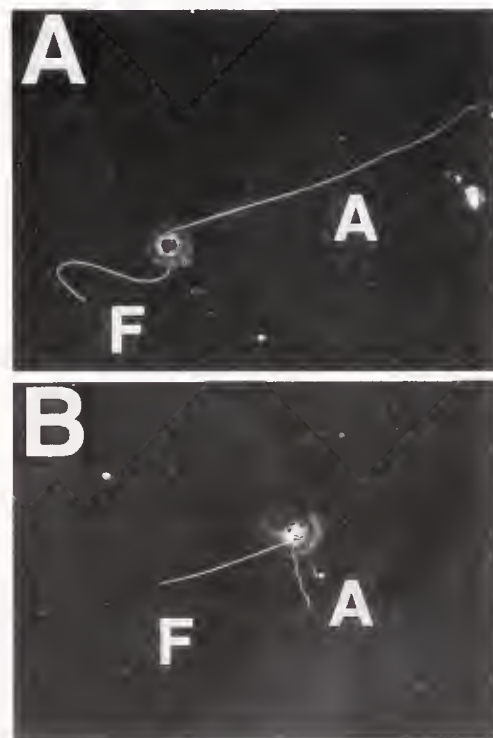


Figure 1. Triton-extracted *Limulus* sperm imaged with the new polarized light microscope equipped with a Nikon PlanApo 60 $\times$ /1.4 NA low strain objective and matching condenser. F, flagellum; A, actin bundle. (A) True discharge. (B) False discharge.

the retardance of bundled microtubules increases linearly with number of microtubules in the bundle. Thus, the retardance of the single actin fiber (R_{single}) can be calculated as:

$$R_{\text{single}} = R/N$$

where R is the measured retardance, and N is the number of

actin fibers (in this case 80 filaments). R_{single} in artificial seawater is estimated as 0.0107 ± 0.0013 nm ($n = 5$). This value is a rough estimate because the actual number of actin filaments in the bundles used in this study was not determined, but the value should be useful for estimating the number of actin filaments contained in cells observed with the new pol-scope.

We are indebted to Dr. L. G. Tilney and S. Inoué for their invaluable suggestions. This work is supported by 1996 Lilli

Fellowship awarded to F.O. and NIH grant (RO1 GM49210) to R.O.

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Reference: *Biol. Bull.* 191: 271–272. (October, 1996)

Mitotic Spindle Fine Structures Observed With Polarized Light

Phong Tran, E. D. Salmon, and Rudolf Oldenbourg (Marine Biological Laboratory)

Recent advances and improvements on the polarizing light microscope have allowed for noninvasive imaging of cellular fine structures with high sensitivity and resolution irrespective of specimen orientation (1, 2). We have used our modified polarized light microscope (pol-scope) to image newt lung epithelial cells during mitosis to examine the dynamic of spindle fine structures.

Primary cultures of newt (*Taricha granulosa*) lung epithelial cells were prepared as described by Rieder and Hard (3). The cells were then imaged with the pol-scope equipped with a Nikon PlanApo 60 $\times$ /1.4NA low-strain objective and a matching Nikon Universal 1.4NA condenser.

Figure 1 shows time-lapsed images of one mitotic spindle. Along with the strongly birefringent arrays of microtubules nu-

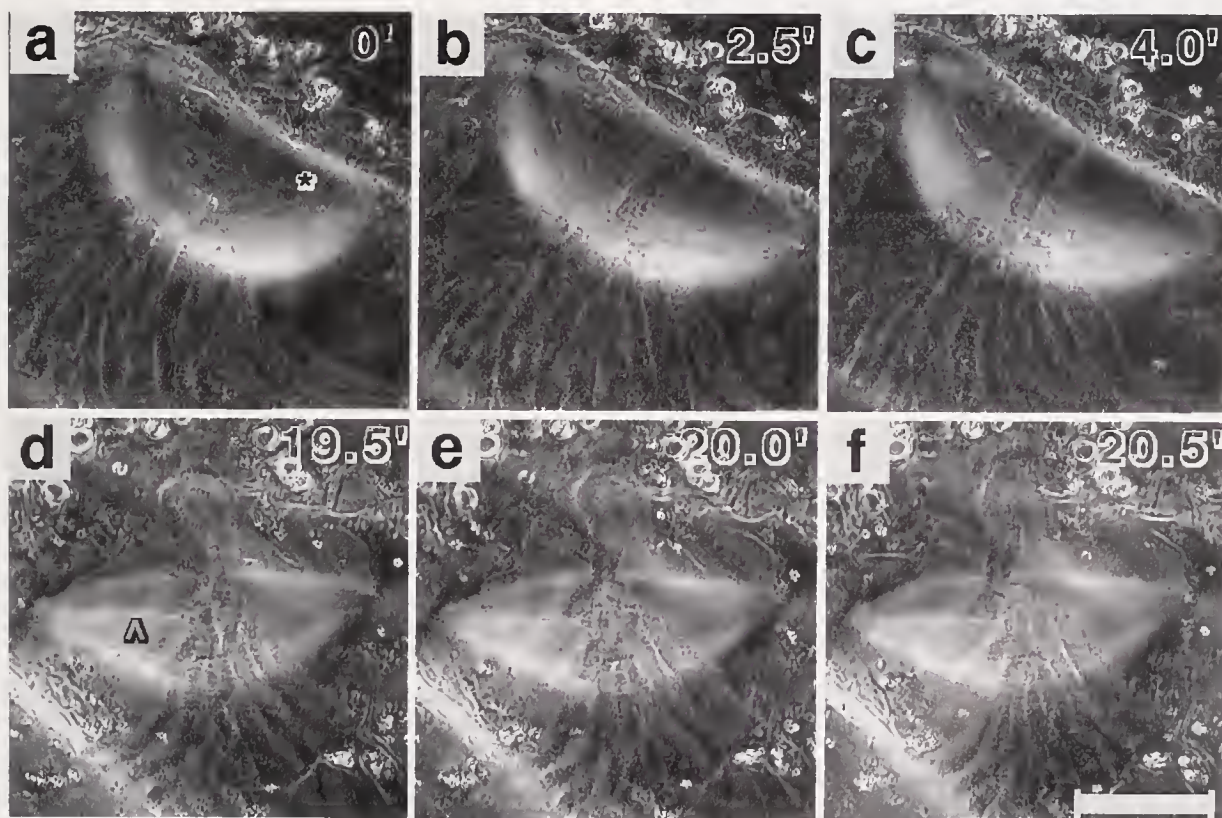


Figure 1. The birefringence of mitotic spindle fine structures imaged with the pol-scope. Sequences a–f depict spindle dynamics over time. The spindle microtubules, chromosomes, and kinetochore fibers are clearly observable. Sequences a–c depict the position of the lone chromosome (*). As the chromosome (*) advances to the mid-zone, the birefringent intensity of the trailing microtubules decreases to match the intensity of the opposite half. Note the indentation impinging on the chromosome arm (b). Sequences d–f depict a kinetochore fiber (A). Slight buckling of the fiber is observable concurrent with the movement of the kinetochore/chromosome away from the pole. Note that the indentation of the kinetochore is visible as the fiber moves away from the pole (d,f), but not as it moves toward the pole (e). Bar = 10 μ m.

cleating at the two centrosomes (poles), weakly birefringent chromosomes are clearly visible. Furthermore, the spindle is dynamic, with the poles as well as the chromosomes constantly changing positions. The birefringence distribution of the microtubules within the spindle is also dynamic. Over time, kinetochore microtubules become visible as highly birefringent fibers directly touching the chromosomes; they can be distinguished from neighboring non-kinetochore microtubules, which are relatively less birefringent. This difference in birefringence implies that the density of microtubules within the kinetochore fibers is higher than that within the rest of the spindle, as also reported by Cassimeris *et al.* (4) using conventional high resolution polarization microscopy, and corroborated by Reider and Bajer (5) using electron microscopy.

Note the lone chromosome moving from a pole toward the spindle mid-zone (Fig. 1a–c). Movement at the cellular scale (*i.e.*, micrometers) has been characterized by Purcell to be dominated by viscous drag rather than mass or inertia (6). Therefore, forces must be constantly exerted to bring about the movement of the chromosome. We observed changes in microtubule birefringence associated with chromosome position. When the chromosome is nearer to one pole than the other, the region between this chromosome and its nearest pole shows a higher microtubule birefringence than the complementary and opposite region. This higher birefringence decreases and will eventually be equalized between opposite regions as the position of the chromosome shifts toward the mid-zone. This observation is consistent with the polar ejection force hypothesis (7), which states that microtubule growth can produce pushing forces.

Note also the marked buckling of the kinetochore microtubule fiber during of the kinetochore away from the pole (Fig.

1d–f). Although the force-generating mechanism that produces buckling—either microtubule-based or kinetochore-based—cannot be determined from the birefringent profile, microtubule buckling requires that there be a force pushing against the ends of the microtubules. The simplest estimate, using Euler's equation for the buckling force on a beam (8), the bending stiffness of a single microtubule decorated with associated proteins [$\sim 5 \times 10^{-23} \text{ Nm}^2$] (9), the average number of microtubules of a newt lung kinetochore [~ 20] (5), and the average measured length of the kinetochore fiber [$\sim 10 \mu\text{m}$], predicts that the buckling force on a 10- μm -long and growing kinetochore fiber would be 100 pN! This gives a ballpark estimate of the pushing forces produced by kinetochore fibers within the cell.

We gratefully acknowledge all members of the MBL Architectural Dynamics in the Living Cell Program for their stimulating discussions, as well as NIH grant GM49210 to R.O.

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Method for Visualizing Filaments in Axoplasm by Electron Microscopy

E. L. Bearer (Brown University)¹, J. Liu^{1,2}, A. Hsu^{1,2}, and T. S. Reese^{1,3}

Three filamentous proteins, actin, tubulin, and neurofilament, are the primary structural components of axons, including the giant axon of the squid, *Loligo pealei*. Organization of these cytoskeletal elements—their length, polarity, and relationship to each other—has been difficult to study because of the thickness of the axon and consequent fixation problems.

Furthermore, the density of the cytoplasm obscures individual filaments in thin-section electron microscopy (EM) (1).

To produce preparations in which individual filaments are partially isolated but still oriented, we have developed a touch preparation (Fig. 1, A–C). Axoplasm extruded from the giant axon (2) onto Parafilm is scored transversely with an eyelash at intervals approximately equal to the diameter of an EM grid. This scoring isolates a thin layer of axoplasm which is then lifted off on an EM grid coated with 1% Formvar and shadowed with carbon. Just prior to touching the axoplasm, grids are glow discharged for 30 s. Grids with adherent axoplasm are immedi-

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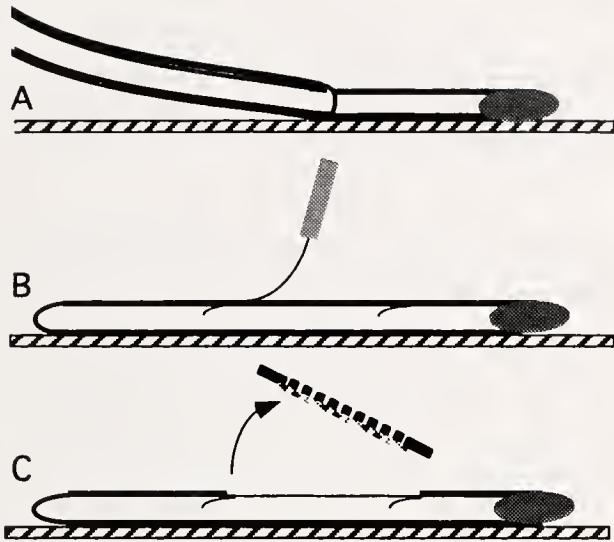


Figure 1. Diagram of the touch prep technique. (A) Axoplasm is extruded onto Parafilm; (B) axoplasm is scored with an eyelash fastened to a glass Pasteur pipette with dental wax; (C) a Formvar-carbon coated, glow-discharged grid is lightly touched to the scored transversely surface of the axoplasm. Immediately afterwards the grid is floated, axoplasmic surface down, on a droplet of buffer and then negatively stained with uranyl acetate. (D) Example of a such a preparation showing microtubules (arrowheads); microfilaments (small arrows), and neurofilaments (large arrow). Bar = 2 μ m.



ately transferred to a 10- μ l droplet of motility buffer (3) and then passaged through three 10- μ l droplets of 1 mg/ml bacitracin and three droplets of 2% aqueous uranyl acetate for 1 min each as a negative stain.

This procedure produces a thin sheet of axoplasm containing microtubules, microfilaments, neurofilaments, organelles, and less structured proteinaceous material. When the preparation is viewed by EM (Fig. 1D), long microtubule bundles and individual microtubules lie parallel to each other, indicating that the procedure does not significantly perturb their original organization, as defined by fluorescence (1) and video microscopy (4). Many long (some $\sim 10 \mu$ m) microfilaments weave among the microtubules and continue beyond their ends. These microfilaments are likely to be actin, although S1 or antibody labeling will be necessary to confirm this identification. Membranous particles associate with both microtubules and microfilaments. Areas between microtubule bundles are occupied by sheets of slightly thicker, straighter filaments with rough surfaces presumably composed of neurofilaments. Also seen was

an unidentified fourth type of filament, short and thinner than the typical microfilaments (not shown).

This procedure thus produces a view of the relationship between the various cytoskeletal elements in the axoplasm with minimal distortion. Such a preparation is suitable for the investigation of the polarity of these actin filaments by S1 labeling, permitting the most definitive determination of which filaments are actin and of the direction of S1 arrowheads by negative staining.

Supported by NIH GMS47368 and CTR 3192 (ELB).

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Localization of Myosin on Tubulovesicular Organelles in the Squid Giant Axon by Immuno-EM

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Organelles in the giant axons of the squid move on actin filaments (1), and the movement involves a barbed-end directed motor (2, 3). Since these initial studies, we have isolated an organelle fraction (4) that retains the ability to move in a motility assay system that uses filaments assembled from muscle actin (5). Motility of these organelles can be reconstituted without the addition of cytosol. The purpose of this study was to determine whether myosin V is associated with these organelles. The organelle fraction was prepared by incubating extruded axoplasm (approximately 5 μ l) in excess axoplasmic

buffer (2 ml) to generate a highly extracted axoplasmic remnant called a ghost (6). The extraction procedure removes soluble proteins, mitochondria and other vesicular organelles, actin filaments and microtubules. When observed by electron microscopy (EM), ghosts are seen to contain primarily two components: tubulovesicular organelles (TVOs) and neurofilaments (NF) (4). A few short microtubule (MT) fragments associated with neurofilaments and TVO complexes were seen also in the ghost (Fig. 1A, panel 3). TVOs have the morphology of smooth endoplasmic reticulum (ER), and we have verified

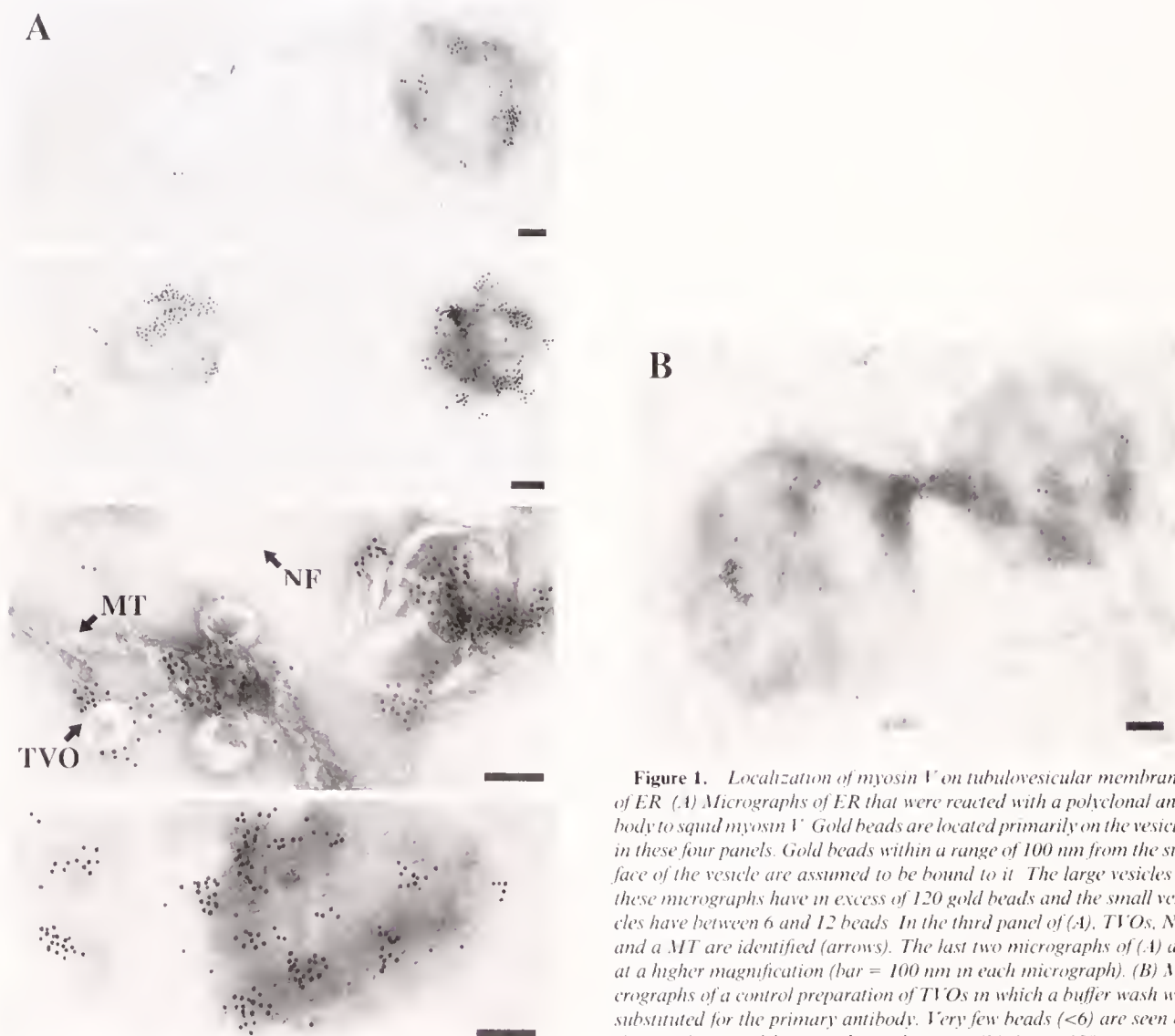


Figure 1. Localization of myosin V on tubulovesicular membranes of ER. (A) Micrographs of ER that were reacted with a polyclonal antibody to squid myosin V. Gold beads are located primarily on the vesicles in these four panels. Gold beads within a range of 100 nm from the surface of the vesicle are assumed to be bound to it. The large vesicles in these micrographs have in excess of 120 gold beads and the small vesicles have between 6 and 12 beads. In the third panel of (A), TVOs, NF, and a MT are identified (arrows). The last two micrographs of (A) are at a higher magnification (bar = 100 nm in each micrograph). (B) Micrographs of a control preparation of TVOs in which a buffer wash was substituted for the primary antibody. Very few beads (<6) are seen on the membranes of the control sample in part (B). Bar = 100 nm.

the identity of these organelles by immuno-fluorescence microscopy, using an antibody to a resident ER protein, protein disulfide isomerase (7, 8).

Immuno-EM was used to detect myosin V on the membranes of these ER vesicles under conditions that support movement on actin filaments. The antibody used in this study was generated in rabbit against myosin V (mol. wt. 196 kD) purified from squid optic lobes (9) by a method shown to isolate myosin V from chick brain (10). The polyclonal antibody to squid myosin V reacts with a single band of 196 kD on immunoblots of squid axoplasm. For localization by immuno-EM, axoplasmic ghosts were fixed with 1% formaldehyde and 0.05% glutaraldehyde in PBS, reacted first with primary antibody to squid myosin V, and then reacted with 10-nm gold-labeled secondary antibody. The gold-labeled ghosts were transferred to a carbon-Formvar-coated grid and stained with uranyl acetate. Control ghosts were prepared by substituting a buffer wash or preimmune serum for the primary antibody step. By the negative staining EM technique, membrane organelles could be positively identified as distinct from the neurofilaments and occasional fragments of microtubule.

When we analyzed the distribution of the 10-nm gold beads in micrographs of preparations reacted with the primary antibody, we found that 85–90% of the gold beads were localized to structures that could be positively identified as membranes (Fig. 1A). The remainder of the gold beads were randomly distributed on the grid surface or on neurofilaments, microtubule fragments or, in a few cases, material that could not be positively identified. The number of gold beads on organelles ranged from 6 for small vesicles to 120 for large vesicles. The number of gold beads on vesicles in the control samples (Fig. 1B) was not above background (usually <5).

On the basis of these data, we conclude that myosin V is localized to the surfaces of ER membranes. Most of the gold beads were localized to the vesicular components of ER, but we cannot rule out the possibility that myosin may be distributed along the tubular membrane elements. These data provide conclusive evidence that myosin is associated with organelles in the squid giant axon and that myosin V is probably the motor for the movement of the organelles on actin filaments.

This work was supported by grants from NSF and NIH and by an MBL Fellowship to J.S.T.

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Potential Role of Syntrophins in Regulating Motility of Sperm in the Sea Urchin *Arbacia punctulata*

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In the sea urchin, sperm motility is regulated by ATP-dependent microtubule motors located in the flagella. The force-generating core structures of the sperm flagella consist of dynein ATPases. Sea urchin dyneins are characterized as light chain (LC), intermediate chain (IC), or heavy chain (HC) by their divergent molecular weights. HC outer-arm dynein is composed of clusters of two to three globular heads connected by fibrous stems to a common base. Recent evidence indicates that LC and IC dyneins are located at the base of these clusters adjacent to the A-tubule. But the specific molecules mediating

the association of dynein to the A-tubule are not well characterized (1–4).

Current studies indicate that syntrophins, a family of 58 kDa membrane associated proteins, are important components of protein complexes that are localized at the mammalian neuromuscular junction (5). Recent reports indicate that defects in the formation of these complexes are associated with Duchenne's muscular dystrophy and may be involved in other muscle disorders (6). We have isolated sperm from the sea urchin *Arbacia punctulata* and have identified a protein of about 58 kD molecular weight that reacts with a monoclonal antibody to syntrophin (Fig. 1) (7). Immune precipitation experiments indicate that this protein associates with IC dynein in resting sperm (Fig. 1, lane 1). Stimulation of sperm motility by

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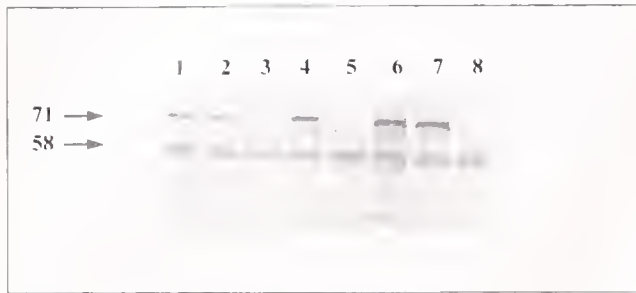


Figure 1. Effects of motility on dynein-syntrophin association. Sperm, freshly retrieved from *Arbacia punctulata*, were lysed and the syntrophin-associated protein complexes isolated by immune-precipitation with a monoclonal antibody to syntrophin (Sigma). The protein complexes were electrophoretically separated on polyacrylamide gels and analyzed for the presence of IC dynein by Western blotting with monoclonal antibodies to *Chlamydomonas reinhardtii* IC dynein (Sigma). Lanes 1–4, unstimulated sperm, lanes 5–8, sperm stimulated with resact (10 nM), lanes 2 & 6, sperm treated with 100 ng/ml cholera toxin, lanes 3 & 7, sperm treated with 10 ng/ml pertussis toxin, lanes 4 & 8, sperm treated with 10 ng/ml botulinum toxin. Dynein and syntrophin appear on the gels with molecular weights of approximately 71,000 and 58,000, respectively.

the egg-derived peptide resact (10 nM) (8) results in the dissociation of IC dynein and this syntrophin (Fig. 1, lane 5).

The motility of sea urchin sperm can be inhibited by bacterial toxins such as cholera toxin and pertussis toxin, which initiate their physiological effects by modulating specific subsets of GTP-binding regulatory proteins (G-proteins) (see reference 9 for a review). Therefore, we next tested the effects of these toxins on sperm retrieved from *Arbacia punctulata*. Using a scale devised by Hansbrough and Garbers *et al.* (10), which evaluates stimulation-induced alterations in sperm swimming patterns and directionality, as well as total movement, we found that both cholera toxin (100 ng/ml) and pertussis toxin (10 ng/ml) inhibit resact-induced sperm motility by 86% and

55%, respectively. In contrast, botulinum toxin (10 ng/ml), a control treatment, had minimal effects on resact-induced motility. We next investigated the effects of these toxins on the composition of dynein-associated immune complexes. In the presence of either cholera toxin or pertussis toxin, syntrophin immune complexes from unstimulated sperm contain both dynein and syntrophin (Fig. 1, lanes 2 & 3). After stimulation with resact, dynein remains associated with syntrophin (Fig. 1, lanes 6 & 7). In the presence of botulinum toxin, dynein continues to dissociate from syntrophin in resact-treated sperm (Fig. 1, lanes 4 & 8). Levels of syntrophin are not altered in cell extracts of either unstimulated- or resact-treated sperm (Fig. 1). Similarly, we used western blotting to determine that dynein levels are not altered (not shown).

Taken together these data indicate that in sea urchin sperm, IC dynein associates with a syntrophin-related 58 kDa protein. In *Arbacia punctulata*, association between IC dynein and this syntrophin is disrupted after stimulation of sperm motility with resact. We speculate that dynein-syntrophin associations may be important in the regulation of sperm motility.

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Reversible Alteration of Molluscan Erythrocyte Morphology by a Natural Hemolymph Activity

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Bivalve molluscs of the genera *Anadara* and *Noctia* have hemoglobin-bearing nucleated erythrocytes with flattened discoidal or ellipsoidal morphology (1, 2). Their cytoskeletal system includes a marginal band of microtubules, and thus they re-

semble non-mammalian vertebrate erythrocytes (3). Unlike vertebrate erythrocytes, however, these cells have an active centrosome associated with the marginal band, making them uniquely interesting (4, 5).

Working with *N. ponderosa* and *A. ovalis*, two species found along the east coast of the United States, we have observed that the erythrocytes become lumpy and spheroidal shortly after re-

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Table I

Characterization of erythrocyte shape transforming activity (X) in cell-free hemolymph (*Noctia ponderosa*, unless otherwise noted)¹

Test #	Hemolymph Treatment	X activity ²	Observations—Initial Conclusions ³
1	none	++	shape alteration (N $\rightarrow$ A) in $\sim$ 5 min
2	freezing (-20°C , -80°C)	++, ++	freeze-thaw stability
3	1:2 dilution (Ringer's)	++	shape alteration (N $\rightarrow$ A) in $\sim$ 5 min
4	1:10 dilution (Ringer's)	+	shape reversal (all A $\rightarrow$ N) in $\sim$ 20 min!
5	boiling bath (30 min)	-, +	high temperature lability
6	three N-cell incubations	-	cells bind or inactivate X (or both)
7	three N-cell ghost incubations	+	ghosts bind or inactivate X (or both)
8	preparation from <i>A. ovalis</i>	++	nonspecificity with respect to genera
9	dialysis vs. Ringer's	++	dialyzable activity
10	filtration: filtrate ⁴	-	
	retentate	+	molecular weight (s) > 10,000
11	protease inhibitor mix ⁵	+, -	may be proteolytic
12	heparin (porcine; 50 U/ml)	++	not vertebrate thrombin-like activity
13	EGTA (10 mM)	-	possibly a Ca^{++} -activated protease
14	EDTA (10 mM)	+	possibly a Ca^{++} -activated protease

¹ Bioassay conditions: $\sim 9 \times 10^7$ erythrocytes/ml (hemacytometer count); frozen-thawed cell-free hemolymph used in all tests except #1; all tests included an untreated control exhibiting ++ activity.

² ++ = >80% A-cells in 5 min (usually >90%); + = 30%–50%; - = <2%.

³ N = normal morphology; A = abnormal morphology.

⁴ Centricon-10 concentrator with 10,000 molecular weight cutoff (Amicon Inc.).

⁵ Aprotinin (10 $\mu\text{g}/\text{ml}$), leupeptin (50 $\mu\text{g}/\text{ml}$), Pefablock (10 $\mu\text{g}/\text{ml}$), pepstatin (50 $\mu\text{g}/\text{ml}$), TAME (10 mM).

moval from the animal (1). A similar phenomenon occurs in *A. trapezia*, the Australian mud ark (6). We can prevent this morphological change by immediate extreme dilution (about 1:200) of the erythrocyte-containing hemolymph into marine molluscan Ringer's solution. Cells of normal, stable morphology (=N-cells) are then retrieved by centrifugation and resuspension in Ringer's.

If N-cells of *N. ponderosa* are resuspended in fresh cell-free hemolymph, > 90% typically assume the abnormal morphology within 5 minutes; we designate these as A-cells. Thus, the hemolymph contains an activity (X) responsible for initiating

the morphological transformation. We have begun characterizing X with two objectives: first, finding a way to inhibit it that is more practical than the current method of extreme dilution; second, determining whether its mode of action is on the cytoskeleton. As an initial approach, we used alteration of N-cell morphology as a bioassay for X activity.

Experiments are summarized in Table I. In each case, cell-free hemolymph was subjected to a particular treatment and X activity was subsequently assayed using fresh N-cells. The activity was stable to freeze-thawing (Table I, #1 vs. #2), permitting use of stored frozen hemolymph in subsequent tests. The results show that the effect of X is concentration dependent and reversible (Table I, #3 vs. #4); cells can recover and reassume normal morphology. The phenomenon is not a simple osmotic response. In the 1:10 dilution (#4), the medium is 90% molluscan Ringer's, yet about 30% of the cells became A-cells before reversal. Since the morphological change might nevertheless involve an osmotic regulatory mechanism, we incubated N-cells in hypo- and hyper-osmotic media to determine whether an osmotic response could mimic that induced by X . The answer was no; in each case, though cells showed evidence of shrinkage or swelling, they retained their general flattened ellipsoidal or circular morphology. In addition, when N-cells and A-cells were lysed in detergent-containing media (Brij), their cytoskeletons retained the general morphological difference. Thus the evidence suggests that the effect involves the cytoskeleton directly.

What is X ? The activity is labile at high temperature (Table I, #5), exhaustible by multiple incubations with N-cells or their ghosts (#6, #7), and nonspecific with respect to genus (#8). It is dialyzable (#9), has molecular weight(s) > 10,000 (#10), can be partially or totally inhibited by a protease inhibitor "cocktail" (#11), and is unaffected by mammalian heparin (#12). Most importantly from a practical standpoint, it is inhibited by EGTA (#13), providing a basis for N-cell production in quantity without dilution. EDTA is less effective (#13 vs. #14), suggesting Ca^{++} -activated proteolytic activity.

Though the hemolymph does not clot, the white cells present become adhesive and form large clumps during the time of erythrocyte shape change in fresh hemolymph. Thus X activity may be part of a molluscan wound-healing mechanism triggered by hemolymph removal.

We are grateful for the support provided, in part, by NIGMS-MBRS GM08176 (J.H., C.T.), NSF MCB9118773, and the NSF REU Program (A.D.).

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Inherent Nonrandom Structure of the Pre-Nuclear Envelope Breakdown Calcium Signal in Sand Dollar (*Echinaracnius parma*) Embryos

Robert B. Silver (Marine Biological Laboratory), Anthony P. Reeves,
Brian P. Kelley, and William J. Fripp

It is widely recognized that calcium plays a key role in controlling cell processes, such as mitosis, although the mechanism through which calcium acts is unresolved. In that role, calcium serves to regulate and coordinate many intracellular processes according to specific patterns in time and space. When considering the “universal” nature of calcium as a second messenger, we find an enigma in that calcium exhibits ubiquity and selectivity as an essential regulator of cell processes, *i.e.* nuclear envelope breakdown (NEB) (*e.g.*, 1–10, 11–15). We are presented with the following question—how can calcium be used to selectively control so many cellular processes?

The pre-NEB calcium signals are seen as transient elevations in the concentration of intracellular free calcium ions (Ca^{2+}). The Ca^{2+} signals are composed of sets of many individual events in which Ca^{2+} increase in local regions—microdomains (defined in 6 and 13–15)—of the cytoplasm. In aequorin-labeled cells the individual Ca^{2+} events are seen as bright observable blobs (BOBs; defined in 13–15) when viewed with a photon-counting video camera and video microscopy techniques (*e.g.*, 13–15). Statistical analyses of such video records show that many of these BOBs occur in nonrandom patterns (14, 15). Sets of three BOBs (*e.g.*, A, B, and C) in which A and B are separated in space-time by the same space-time displacement (velocity) as B and C—and that all reside on a common vector—are said to be linked (13–15); linked BOBs are thus nonrandom and are defined as quantum emission domains, or QEDs (14, 15). We hypothesize that the enigma of Ca^{2+} signaling (*e.g.*, in mitosis), typified by its concurrent universality and selectivity and specificity, is resolved through the expression of structured, nonrandom space-time patterns of discrete Ca^{2+} , QEDs acting within Ca^{2+} microdomains.

Transient elevations in Ca^{2+} concentration associated with cell-cycle events were detected with a photon-counting video camera as BOBs in second-cleavage sand dollar (*Echinaracnius parma*) blastomeres cultured in Ca^{2+} -free Jamarin artificial seawater (Jamarin Laboratory, Inc., Osaka, Japan; 11, 12, 16). The photon-counting camera used in this study was a specially constructed dual-microchannel plate intensified saticon device (C-2400-20 from Hamamatsu). This detector operates as follows: when incident photons strike the faceplate of the intensifier, a cloud of electrons proportional to that number of photons is produced in the microchannels and presented to the faceplate of the saticon camera. A 20 $\times$, 0.75 NA plan-Apo objective lens was used for these observations. The blastomeres were injected with 10-pl doses of semisynthetic aequorin (17–19) shortly after the first cytokinesis following fertilization. The injected blastomeres were observed by multispectral video microscopy, including differential interference contrast (DIC) optics in the dark at 14°C. Two-cell embryos provide one experimental and one (sister) control cell; in the absence of experimental manipulation their activities would be identical (11, 12, 16). More-

over, since blastomeres in the second cell cycle are free of the effects of fertilization activation, they more closely resemble subsequent cell cycles (11, 12, 15, 16). Sand dollar embryos are well suited to these experiments: they are nearly transparent, and they undergo the early cell cycles with near-perfect synchrony. Video records of aequorin-labeled sand dollar cells were analyzed with a number of newly developed computational tools to determine if Ca^{2+} -QED structure existed.

Analysis and modeling of BOB and QED patterns show that (1) pre-NEB signals consist of large, structured populations of BOBs arising from the perinuclear region; (2) pre-NEB BOB/QED patterns have inherent, nonrandom space-time structure; (3) no inherent structure is detected in three-dimensional uniform, random populations of events generated in models or empirically; (4) linked Ca^{2+} , QEDs occur in perinuclear microdomains; (5) space-time backpropagation of the BOB/QED velocity vectors demonstrate that there is no diffusion-related component such as an agonist-induced or calcium-induced calcium release acting at distances $> 1 \mu\text{m}$ in these signals. The time and duration of these Ca^{2+} signals coincide with the period when cells require Ca^{2+} signals to accomplish NEB and subsequent mitotic steps. The signals appear to be digital in nature, and encoded as frequency modulation or pulse code modulation. From other work (*e.g.*, 6, 21–27), it appears that this signal mechanism is not unique to mitotic cells. The deterministic nature of these signals indicates that the enigma of Ca^{2+} signaling is resolved through structure inherent in these signals, supporting our concept.

Research grant support from NSF to R.B.S. (MCB-9308024) and gifts of semisynthetic aequorin preparations from Dr. Osamu Shimomura (Marine Biological Laboratory) are gratefully acknowledged. Helpful interactions with Emilio Stromboli and others during the course of this study are also gratefully acknowledged. The authors also thank the reviewers for their generous assistance in the reviewing process.

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Reference: *Biol. Bull.* **191**: 279–280. (October, 1996)

Intracellular pH Measurements During Fertilization of Surf Clam (*Spisula solidissima*) Oocytes François Dubé (Université de Montréal) and William R. Eckberg¹

Previous work indicates that surf clam (*Spisula solidissima*) oocytes undergo increases in both intracellular Ca^{2+} and pH upon activation. However, direct measurements of both ionic responses in live oocytes, following fertilization, are still lacking. We measured intracellular pH (pH_i) in 2',7'-bis(carboxyethyl)-5(6) carboxyfluorescein (BCECF)-loaded oocytes monitored with a Zeiss Attofluor digital fluorescence imaging microscope.

Oocytes were loaded with BCECF-AM (5 μM) for 15 min and washed in MBL artificial seawater containing 5 mM TRIS-HCl, pH 8.2. Groups of oocytes were put on polylysine-coated coverslips attached to petri dishes and their fluorescence signals at 520 nm were periodically (6-s intervals) measured for excitation wavelengths set at 430 and 488 nm. Fluorescence intensity ratios (488/430, FIRs) were thus monitored as indicators of pH_i .

The addition of 5 mM ammonia induced immediate large increases in FIRs that rapidly, within seconds, reached a new stable level and then decreased slowly with time (Fig. 1). The addition of sperm to oocytes caused slow increases in FIRs, after a varying lag period (1–2 min). FIRs in fertilized oocytes steadily increased over a 10-min period, after which they remained stable for extended periods of time (>20 min, Fig. 1). The addition of 50 mM KCl to oocytes caused immediate increases in FIRs that leveled off within 5 min to values similar to those of fertilized oocytes (Fig. 1). The pH shift in fertilized or KCl-activated oocytes was completed before germinal vesicle breakdown (GVBD) and amounted to about half of the upward shift seen after ammonia addition that did not induce GVBD. According to an *in vitro* calibration curve, resting pH_i of surf clam oocytes is about 6.82. Ten minutes after fertilization, the pH_i goes up to 7.24 for an average rise (ΔpH_i) estimated as 0.42 pH units. KCl caused a similar rise in pH_i ($\Delta\text{pH}_i = 0.33$), whereas 5 mM ammonia raised it initially to much

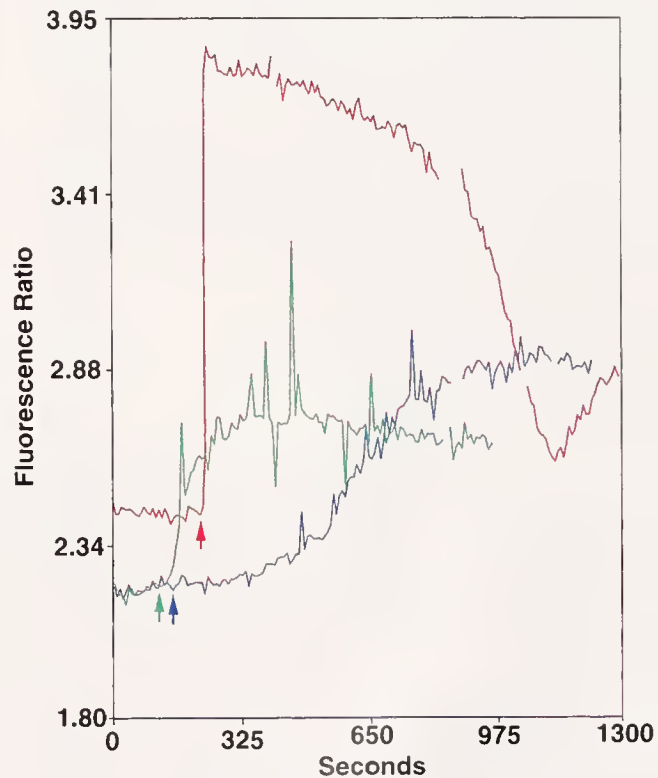


Figure 1. Intracellular pH changes measured in BCECF-loaded surf clam (*Spisula solidissima*) oocytes: the effects of fertilization and additions of KCl or ammonia. Red line: Ammonia (5 mM) was added at the arrow. No germinal vesicle breakdown (GVBD) occurred during the experiment. Average readings from 6 oocytes. Blue line: Normal fertilization with sperm added at the arrow. All oocytes underwent GVBD. Average readings from nine oocytes. Green line: Artificial activation by KCl (50 mM) added at the arrow. All oocytes underwent GVBD. Average readings from nine oocytes.

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higher values ($\Delta\text{pH}_i = 0.78$). Nevertheless, it is known that ammonia concentrations higher than 10 mM are required to induce GVBD in this species (4), which strongly suggests that the activating effect of such high ammonia concentrations is not solely related to its capacity to raise pH.

On the basis of these experiments, it appears that an increase of pH_i occurs after fertilization or artificial activation of surf clam oocytes. Apparently, however, this upward pH shift is not sufficient for the onset of GVBD.

We thank Peter J. S. Smith for access to the Attofluor micro-

scope and Kasia Hammar for assistance in using it. Supported by an NSERC-Canada grant to F.D., a Council for Tobacco Research grant to W.E. (#3378), and an NCRR (P41 RR01395) grant to P.J.S.S.

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Effects of Altering pH_i and pH_o on the Activation of *Chaetopterus* Eggs

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Chaetopterus eggs are shed at prophase of meiosis I and undergo germinal vesicle breakdown (GVBD) upon contact with natural seawater (NSW). The initiating agent in NSW is unknown, but it must be a trace component because no artificial seawater formula is able to induce GVBD (1). After GVBD

they form a spindle and arrest at metaphase until they are fertilized or artificially activated. If fertilized, they complete meiosis, begin cleavage and develop into larvae; if artificially activated with 50-100 mM excess K⁺, they complete meiosis and undergo differentiation without cleavage (2).

Metabolic activation after fertilization of sea urchin eggs involves increases in cytoplasmic free Ca²⁺ and pH (pH_i) (3, 4). While increases in intracellular free Ca²⁺ seem to be universally involved in egg activation, the role of pH_i is controversial. In *Spisula* the pH_i increases after fertilization, but this increase is evidently not an important signal in the initiation of development (5, 6). Furthermore, the pH_i does not increase in starfish eggs at maturation or fertilization (7, 8). Accordingly, we have investigated the possible role of pH_i changes at oocyte maturation and egg activation in *Chaetopterus* by two approaches. We tested the effects of reduced extracellular pH (pH_o) and NH₄Cl on GVBD and egg activation, and we measured changes in intracellular pH using fluorescence microscopy.

We performed several experiments to test the effects of changing pH_o and pH_i on GVBD and egg activation. We first tested the ability of NSW (buffered with 10 mM each of HEPES and PIPES) to induce GVBD at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.2 (the normal pH of seawater). GVBD was normal at all pH_o tested. In NSW buffered at pH 6.0 with 10 mM Na-

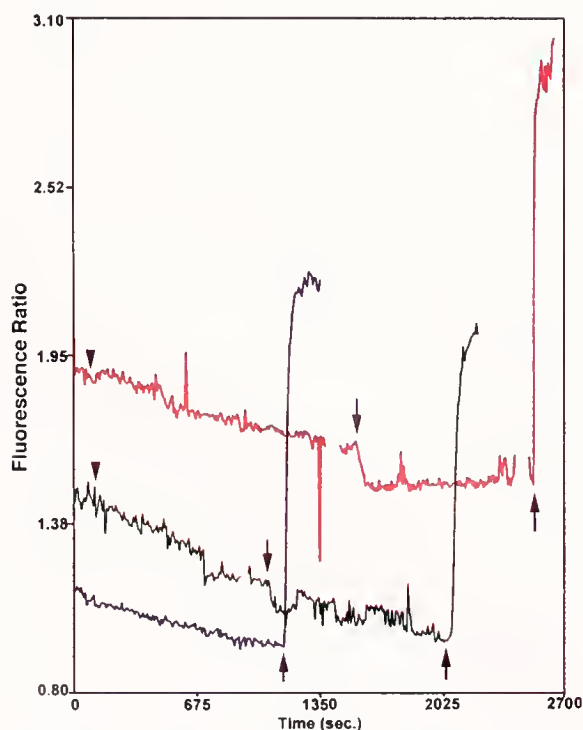


Figure 1. Fluorescence ratio (430 nm and 488 nm excitation, 520 nm emission) of 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) in *Chaetopterus* oocytes during GVBD and egg activation. Oocytes were loaded with 5 μM BCECF-AM (a cell-permeant acetoxy-

methyl ester of BCECF) for 15 min at room temperature and washed twice with CaFASW and attached to polylysine-coated cover slips for observation by epifluorescence. Downward traces indicate acidification of the cytoplasm; upward traces indicate alkalinization. The small peaks are machine "noise." The red (5 oocytes) and green (2 oocytes) traces show the results from two different experiments. The blue trace represents a control experiment in which unstimulated oocytes were left in CaFASW. Arrowheads indicate the time of addition of natural seawater to trigger GVBD, downward-pointing arrows indicate the time of addition of 100 mM KCl to activate the eggs; upward-pointing arrows indicate the time of addition of 10 mM NH₄Cl which was added at the end of each experiment to ensure that alkalinization of the cytoplasm could have been detected had it occurred.

acetate, germinal vesicles disappeared, but spindles failed to form, and therefore, this is not a true progression to M phase. Next we treated oocytes with NH_4Cl , pH 8.2, which directly increases pH_i (9). However, concentrations of NH_4Cl up to 10 mM in artificial seawater failed to induce GVBD. At 20 mM NH_4Cl germinal vesicles disappeared, but, as with acetate-buffered seawater, spindles failed to form. Third, we tested NH_4Cl on metaphase-arrested oocytes. Concentrations up to 0.5 mM had no effect. Higher concentrations caused elevation of some fertilization envelopes in a dose-dependent manner. In *Chaetopterus* oocytes fertilization envelope elevation results from cortical microvillar elongation (10). At 10 mM NH_4Cl , some oocytes became ameoboid, indicating the onset of pseudocleavage, a sign of differentiation without cleavage; at 20 mM, approximately 20% of the oocytes formed protrusions at the animal pole, which resembled attempts at polar body formation. By 90 min after activation, many oocytes in 20 mM NH_4Cl had polar bodies, and some were ameoboid; a few in 10 mM NH_4Cl also had polar bodies and were ameoboid. However, at these high concentrations, NH_4Cl raises the pH_i to unphysiological levels (Fig. 1) and may have other effects as well (11), so it would be unwise to attribute this egg activation solely to increased pH_i .

We used the Attofluor digital microscope to perform experiments to examine whether the pH_i changes during GVBD or egg activation (Fig. 1). pH_i underwent a slow downward drift during GVBD. We do not believe that this represents acidification of the cytoplasm associated with GVBD, as a similar drift was observed in oocytes not undergoing GVBD. Addition of KCl to a final 100 mM activated eggs as evidenced by polar body formation. This involved a brief, more rapid, decrease

followed by stabilization of pH_i . Addition of NH_4Cl , pH 8.2, to a final 10 mM resulted in a rapid and substantial alkalization of the cytoplasm. As shown above, this treatment did not elicit GVBD in prophase-arrested oocytes and only weakly elicited polar body formation in metaphase-arrested oocytes.

We conclude that the pH_i changes little, if at all, during GVBD and after egg activation in this organism and that pH_i has no obligatory role in GVBD or egg activation.

We thank Dr. Peter J. S. Smith for access to the Attofluor microscope, supported by NCRR (P41 RR01395). W.R.E. was supported by grants from the NIH (GM08016) and the Council for Tobacco Research, USA, Inc. (#3378). F.D. was supported by an NSERC-Canada grant.

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Gradual Loss of a DNA-Inducible Protein Kinase Activity From the Cytoplasmic Extracts of *Arbacia* Embryos

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The DNA-dependent protein kinase is a serine/threonine protein kinase that requires nicked double-stranded (ds) DNA for its activity (1). It plays a crucial role in DNA repair (2), but its role in development is not clear. DNA-activated protein phosphorylation has been reported in extracts from cultured human cells and the oocytes or the embryos of several marine invertebrates (3). Herein, we show that the cytoplasmic extracts of sea urchin (*Arbacia punctulata*) eggs contain a kinase that is inducible by exogenous dsDNA only after fertilization and is not detectable in cytoplasmic extracts prepared from blastulae, gastrulae, and plutei.

Homogenate-catalyzed DNA-dependent phosphorylation assays performed in cytoplasmic extracts of eggs and embryos

demonstrate that unfertilized eggs lack the DNA-inducible kinase activity (Fig. 1). However, the enzyme activity is seen in extracts from fertilized eggs. A gradual decline in the cytoplasmic enzyme activity is observed through 50 (first cleavage) and 103 (second cleavage) minutes post-fertilization. Cytoplasmic extracts prepared from the blastulae, gastrulae, and plutei also lack the DNA-inducible phosphorylation activity. Unfortunately, we could not perform a meaningful enzyme assay in whole cell extracts because cell sonication generates nicked nuclear DNA. For this reason, the control reaction in the absence of DNA could not be performed in these extracts since α -casein is a non-specific substrate for DNA-dependent protein kinase and can be phosphorylated by other kinases.

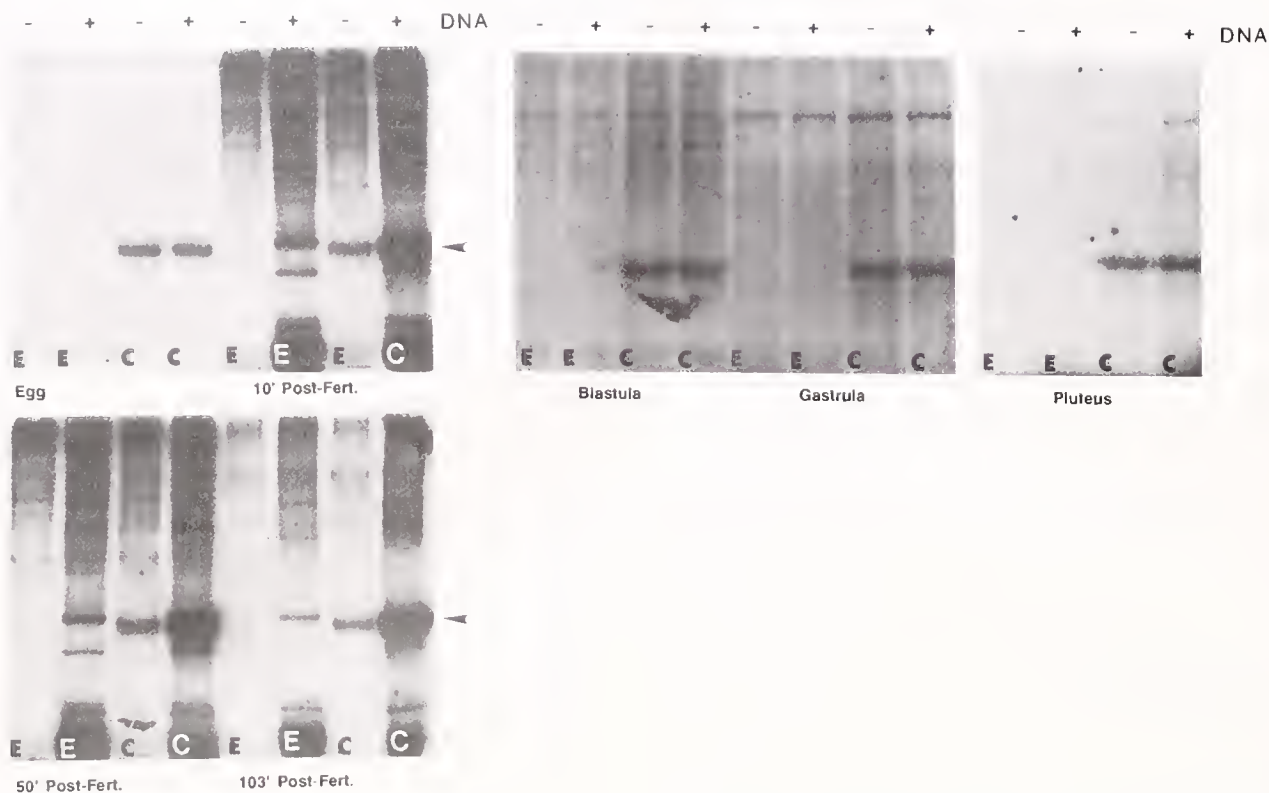


Figure 1. Autoradiogram shows variation in DNA-dependent protein phosphorylation activity during embryonic development. Cytoplasmic extracts from eggs and embryos were prepared as already reported (4). A 15 μ l reaction required 10 μ l of cytoplasmic extract, 75 ng of sonicated calf thymus DNA, 15 μ g dephosphorylated α -casein (Sigma) as substrate, 2 mM $MgCl_2$, and 130 μ M ATP. The reaction was carried out at 15°C, and started by adding 10 μ Ci of [γ - ^{32}P]-ATP (6000 Ci/mmol) (NEN, Du Pont). The phosphorylation reaction (1 μ l) was analyzed on a 10% polyacrylamide gel (SDS-PAGE). E—extract only (without any exogenous substrate); C—extract with exogenous α -casein added as an exogenous substrate. Arrows indicate the position of phosphorylated casein. Absence and presence of dsDNA in the extract are indicated by — and +, respectively.

Therefore, a specific substrate is needed to assay for the specific enzyme activity in whole cell extracts.

These results demonstrate the presence of a DNA-dependent protein kinase in the cytoplasm of eggs and embryos of sea urchin. The rapid appearance of activity in the cytoplasmic extracts from fertilized eggs could be due to either the synthesis of the enzyme itself or the activation of preexisting inactive enzyme by post-translational modification. However, we can not rule out the possibility that the enzyme is nuclear in unfertilized eggs and is released after fertilization.

We thank Dr. Carl Anderson and Dr. William Dynan for

helpful discussions. Grant support came from NIH (AR 32549 to JAH), Mason Trust, the Arthritis Foundation.

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A DNA-Inducible Kinase Activity Undergoes a Change in Cellular Localization During Development in *Arbacia*

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DNA-dependent phosphorylation has been detected in various cell types from a wide range of organisms. The enzyme responsible for this activity in human cell lines, DNA-dependent protein kinase (DNA-PK), has been well characterized. Human DNA-PK is composed of two subunits—a regulatory subunit (Ku) and a catalytic subunit (p460, previously known as p350) and has been shown to have roles in transcription, recombination and repair of DNA (1–4).

A DNA-dependent kinase activity has previously been found in crude extracts from fertilized sea urchin eggs (5, 6). *In vitro* kinase assays have shown that this activity is present in cytoplasmic extracts of fertilized *Arbacia* eggs only up to the 4-cell stage (6). In this report we further examine the cellular localization of DNA-dependent kinase activity in urchin development.

We performed *in vitro* DNA-dependent kinase assays using a specific peptide derived from p53—a known substrate of hu-

man DNA-PK (7). This enabled us to compare the relative levels of activity in both cytoplasmic and whole cell extracts from different stages of development. As in the assays performed with α -casein as a substrate (6), we were unable to induce the kinase activity in extracts from unfertilized eggs, although a high level of DNA-inducible activity is present in both cytoplasmic and whole cell extracts of fertilized eggs (Fig. 1a). This activity gradually decreases in cytoplasmic extracts of 2- and 4-cell stages (up to 103 min post-fertilization) and is not detectable in cytoplasmic extracts from the blastula stage onward. Sonicated whole-cell extracts from all stages post-fertilization did, however, contain high levels of inducible kinase activity. This result suggests that the cytoplasmic activity in the fertilized egg becomes localized to the nuclear fraction in embryos at later stages.

A monoclonal antibody (mAb 42-26) raised against p460, the catalytic subunit of human DNA-PK (2), was used to probe western blots of cytoplasmic extracts from eggs and developing embryos. The serum recognizes a protein of ~460 kDa in both

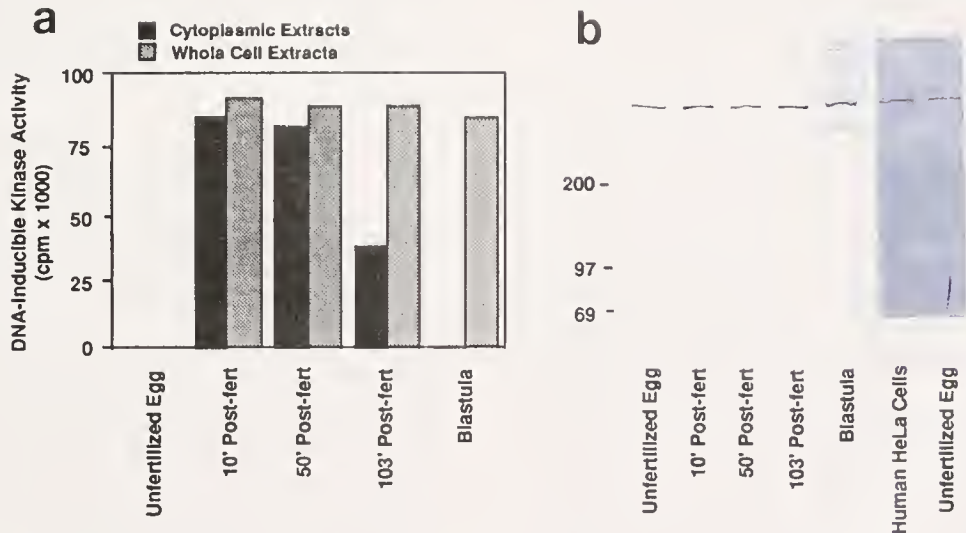


Figure 1. (a) DNA-dependent kinase activity of cytoplasmic and sonicated whole cell extracts from different stages of *Arbacia* development. Kinase reactions were set up exactly as in (6) except that a specific peptide derived from p53 (EPPLSQEAFADLWKK) was used as substrate (8). Reaction products were spotted onto p81 phosphocellulose paper, which was washed successively with 30% acetic acid, 15% acetic acid, and then 5% acetone. The 32 P incorporated on the dried filters was quantitated by scintillation counting. (b) Immunoblot analysis of extracts made from eggs and embryos of *Arbacia* (prepared as in (7)). Extracts were run on a 7.5% SDS gel, transferred to nitrocellulose, and processed for immunoblotting using a monoclonal antibody (mAb 42-26) against human p460 and an anti-mouse alkaline phosphatase-conjugated secondary antibody. The position of molecular weight markers in kDa are indicated on the left. (c) *In situ* whole-mount immunostaining of *Arbacia* eggs and embryos using mAb 42-26. Unfertilized eggs (A and B), 2- to 4-cell embryos and morula (C and D) were immobilized on poly-L-lysine coverslips and fixed in 3.7% formaldehyde, 0.05% NP-40 in PBS for 8 min. After incubation in blocking solution (10% goat serum/1% BSA in PBS) for 30 min at room temperature, the coverslips were treated with either mouse pre-immune IgG (A and C) or with mAb 42-26 anti-p460 (B and D), each at 1:50 in the block solution for 30 min at room temperature. After washing in PBS, the coverslips were incubated with a fluorescein-conjugated goat anti-mouse secondary antibody (1:64; Sigma) for 30 min and then washed again in PBS before being viewed with immunofluorescence. Scale bars: A and B, 27 μ m; C and D, 14 μ m.

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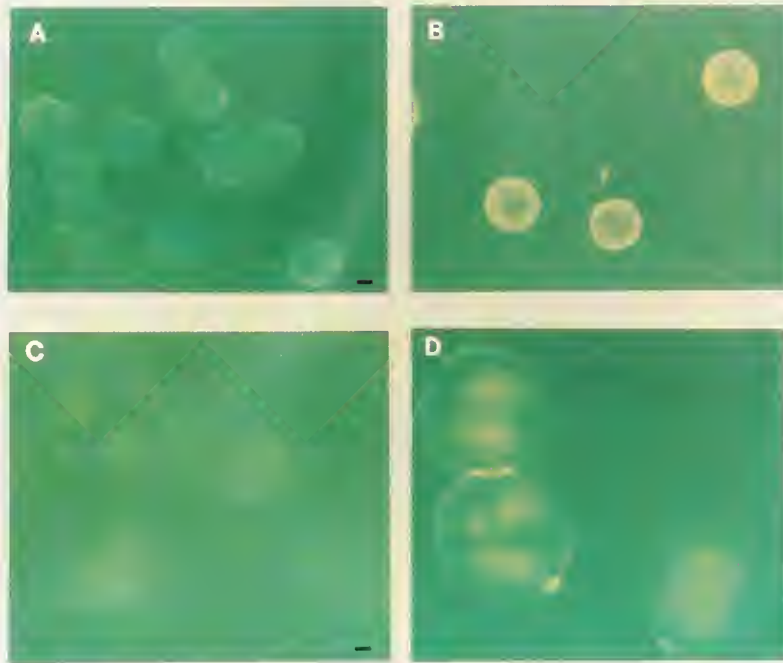


Figure 1. (Continued)

unfertilized eggs and early embryos of all stages as well as protein of similar size in human HeLa cell extracts (Fig. 1b). This protein is thought to represent the urchin homolog of the catalytic subunit of DNA-PK.

Lastly, we used the anti-human p460 monoclonal serum to immunostain *Arbacia* eggs and embryos (Fig. 1c). The urchin p460-related protein appears to be exclusively cytoplasmic in unfertilized eggs and then undergoes a change in localization to the nuclei in cleaving embryos.

These results confirm the presence of a DNA-dependent protein kinase activity in fertilized urchin eggs. *In vitro* kinase assays reveal that the localization of this activity changes from the cytoplasm in fertilized eggs to the nuclei in later stages of development. A protein related to human p460 is present in all stages of *Arbacia* development and may represent the catalytic subunit of urchin DNA-PK. The change in localization of this p460-related protein from the cytoplasm to the nuclei after fertilization is consistent with the change in localization of DNA-inducible kinase activity in specific *in vitro* kinase assays. Although the role of the DNA-PK activity in *Arbacia* development is not yet known, we suggest that the enzyme is activated upon fertilization and may be required during the early rapid cell cycles in embryogenesis.

Supported by a grant from NIH (AR 32549) and an award from the Mason Trust. J.A.W. was supported by Miller and Associates Fellowship (1996) from the MBL. We thank Drs. T. Carter and W. Dynan for providing the antibodies and helpful discussions.

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Modified Spiral Cleavage: The Duet Cleavage Pattern and Early Blastomere Fates in the Acoel Turbellarian *Neochildia fusca*

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Spiral cleavage, which characterizes the embryos of phyla belonging to the Spiralia, typically involves the generation of four quartets of micromeres from the four macromeres produced by the first and second cleavages. The direction of the cleavage planes forming the micromere quartets alternates between dextrotropic (clockwise) and laeotropic (counterclockwise), with the first quartet usually resulting from a dextrotropic division to

the eight-cell stage. Cell lineage studies of embryos with quartet spiral cleavage demonstrate that the first three quartets form ectoderm (1).

Embryos of the acoel turbellarian Platyhelminthes exhibit a modified form of spiral cleavage in which only two macromeres form before pairs or duets of micromeres are generated beginning at the four-cell stage (Fig. 1a, b). However, the few studies on acoel development are in disagreement as to the direction of the cleavage planes during micromere formation, and there have been no lineage analyses since the 1909 work of Bresslau (2). We have examined the cleavage pattern and fates of the first three micromere duets in the embryo of the acoel *Neochildia fusca*.

Adult specimens of *N. fusca* were collected from Sippewisset

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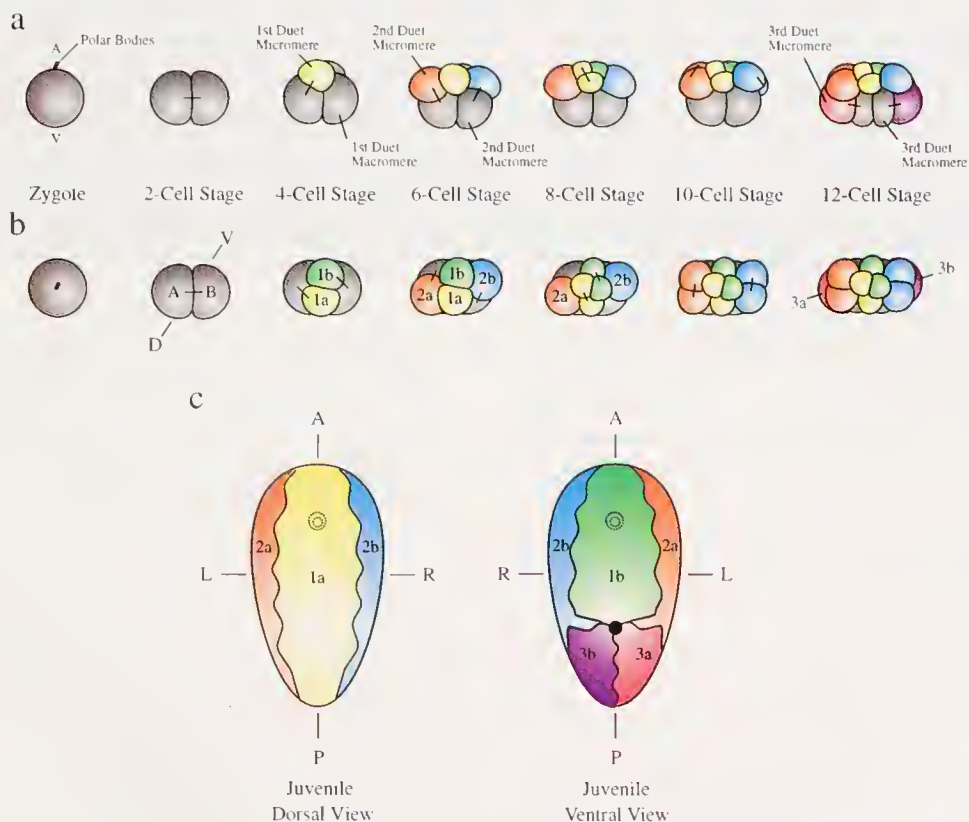


Figure 1. The duet cleavage pattern and ectodermal fates of the first three duets of micromeres in *Neochildia fusca*. (a) The cleavage pattern as seen from the side. (b) Animal pole view of cleavage. Duet spiral cleavage is characterized by the formation of pairs of micromeres (duets) which are generated from the vegetal macromeres after the two-cell stage. The four-cell stage results from a laeotropic division of the macromeres to form the first duet (1a and 1b). The second duet (2a and 2b) is generated by another laeotropic division to the six-cell stage. The 8- and 10-cell stages are characterized by dextrotropic divisions of the first duet and second duet respectively. The third duet (3a and 3b) is produced by an only slightly laeotropic division of the macromeres to form the 12-cell stage. (c) An idealized cell lineage of the micromere duets, with the color and nomenclature of each ectodermal domain corresponding to that of the micromere from which it is generated. These ectodermal domains represent a composite of 63 cases in which the actual boundaries varied to some degree from animal to animal. The small double circle in the anterior region of the worm symbolizes the statocyst; the dark circle located in the posterior region in the ventral view indicates the mouth.

Marsh and maintained in bowls of seawater where fertilized eggs were deposited in masses among sand grains. Fertilization is internal and at deposition the first cleavage spindle has already formed. Embryos were removed from their egg shells with sharp forceps, and individual blastomeres were injected with 2 to 4 drops, depending on blastomere size, of the fluorescent lineage tracer DiIC₁₈ (Molecular Probes, Inc., Eugene, OR) according to the method of Martindale and Henry (3). DiI is a lipophilic dye that binds to membranes and remains in the injected blastomere and its progeny. The embryos were raised at 22°C and the juvenile worms examined for labeling patterns at five days.

The cleavage pattern of *N. fusca*, shown in Figure 1a, b, demonstrates that each duet of micromeres is actually generated by a laeotropic division of the macromeres, while divisions of the micromeres occur in a dextiotropic direction. This is in contrast to the reports of Bresslau (2) and Costello (4) that the cleavage producing the second duet is dextiotropic, but in agreement with Apelt who examined 13 acoel species (5). (Note that the spiral character of cleavage diminishes at third duet formation, which tends to be almost bilateral). Bresslau (2) indicates that a fourth duet forms internally after gastrulation.

When one blastomere was injected with DiI at the two-cell stage in seven embryos, four juvenile worms were labeled on the left-dorsal side and three on the right-ventral side. Figure 1c is an idealized summary diagram of the results of labeling one blastomere of each duet up to the 12-cell stage (exact boundaries of the ectodermal domains varied among the embryos), and shows that all three duets give rise to ectoderm. When one first duet micromere was injected in 24 embryos, 14 juvenile worms showed a characteristic mid-dorsal ectodermal label from anterior to posterior and 10 a mid-ventral label that extended from the anterior end to the mouth. In 21 cases in which a second duet micromere was marked, a lateral patch of ectoderm extending from the boundary of the mid-dorsal label to the boundary of the mid-ventral label was fluorescent on the left side in 10 cases and on the right in 11. Injection of a third duet micromere in 18 embryos produced a left-posterior-ventral label in 10 cases and a right-posterior-ventral label in 8 cases.

Our results show that acoel development differs from that of quartet-cleaving species, not only in the formation of ducts rather than quartets of micromeres, but also in the absence of alternating cleavage, and in the plane of first cleavage, which divides the embryo into left-dorsal and right-ventral halves. This is in contrast to most quartet-cleaving species, where first cleavage produces left-ventral and right-dorsal halves. The only other reported example of a laeotropic division forming the first set of micromeres is the counterclockwise cleavage to first quartet in some sinistral species of gastropod molluscs which produce a left-handed shell (1).

It is likely that the duet cleavage pattern is derived from an ancestral quartet cleavage program shared by the majority of spiralian, but it is not clear how this transition was accomplished. One explanation is that duet cleavage may have arisen via a heterochronic change in the timing of micromere formation, perhaps by suppression of second cleavage. However our results indicate that this is not likely. Even if second cleavage were skipped, one would still expect the first micromeres to form dextiotropically. The fact that the first three duets are generated by laeotropic divisions, that four rounds of micromeres are formed, and that the first three duets continue to generate ectodermal derivatives, with no precocious appearance of mesoderm or endoderm, argue for a more radical alteration of the spiralian program.

The authors thank the generous community of the Marine Biological Laboratory. B.C.B. was supported by the Union College Faculty Research Fund. J.Q.H. was supported by the UIUC. M.Q.M. was supported by NSF, the Evelyn and Melvin Spiegel Endowed Fellowship Fund and the Bernard Davis Fund.

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Reference: *Biol. Bull.* 191: 286–288. (October, 1996)

The Origins of Mesoderm in the Equal-Cleaving Nemertean Worm *Cerebratulus lacteus*

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The spiralian comprise a large group of protostome invertebrate phyla that display a highly conserved pattern of development. In particular, remarkable similarities in their embryonic cleavage pattern (spiral cleavage) and cell lineages have been observed (1, 2). For instance, in annelids, molluscs, and polyclad turbellarians, the mesoderm consistently arises from two

distinct sources (3). Some mesoderm is derived from specific second, third, or both quartet micromeres that also generate ectodermal fates and are therefore referred to as “ectomesodermal” cells. They give rise to what has been considered as “larval mesoderm” in forms that develop via a feeding larval stage. Additional mesoderm is derived from the fourth quartet micromere (4d) of the dorsal, D cell quadrant. This cell, which also contributes to the formation of endoderm, is referred to as the “mesentoblast” and has been considered the source of the “adult mesoderm.”

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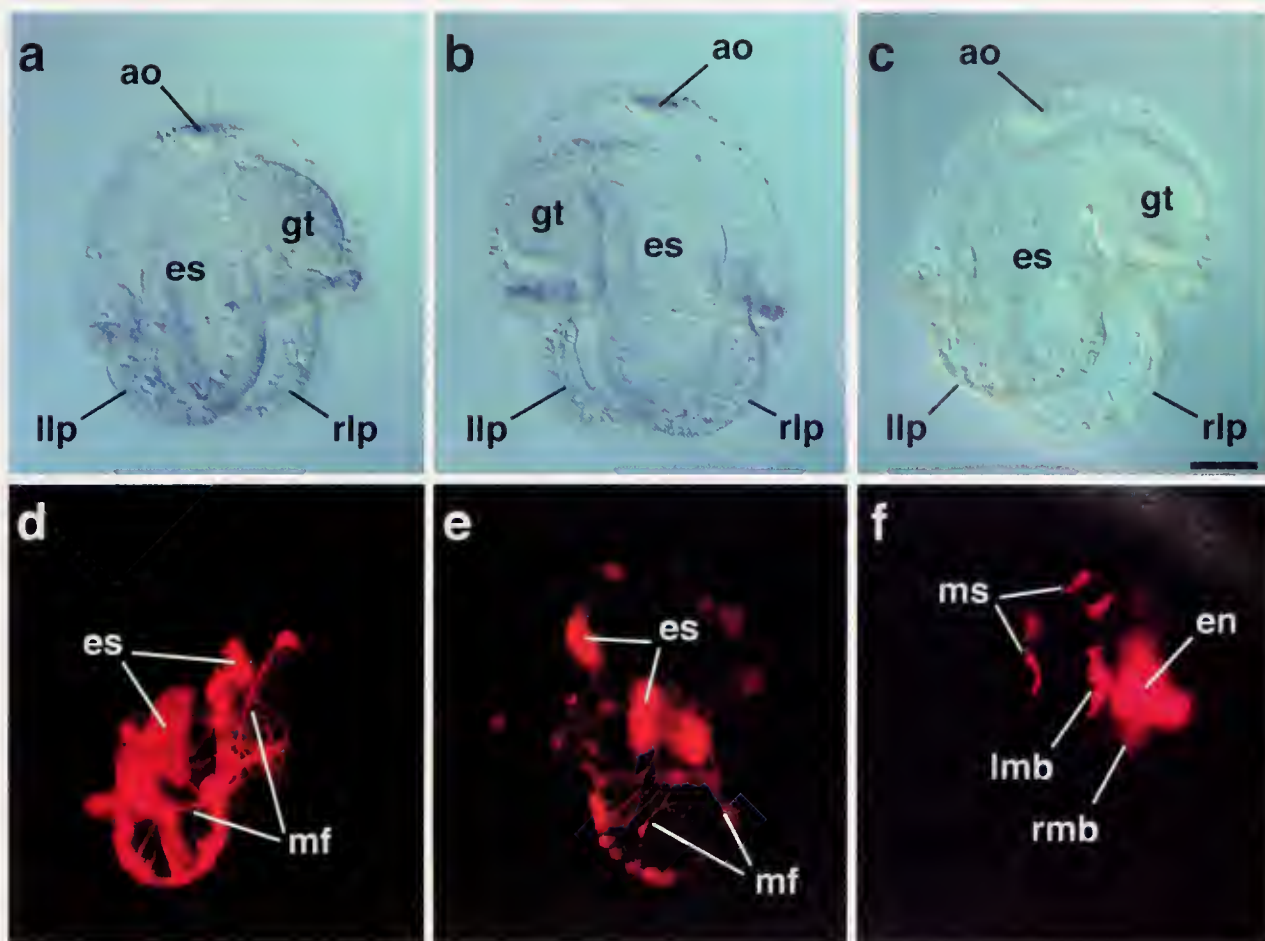


Figure 1. Differential interference (a, b, c) and fluorescence photomicrographs (d, e, f) revealing the larval progeny of specific micromeres in the embryos of *C. lacteus*. Red color depicts the presence of microinjected DiI lineage tracer within the derivatives of these cells. The larvae shown in a, d and c, f are viewed from the left side, while the larva shown in b and e is shown from the right side. In all cases the apical organ (ao) is directed upwards. (a) DIC images of the larva; the third quartet micromere of the A cell quadrant (3a) had been injected. In the corresponding fluorescence micrograph (d), a large number of labeled muscle fibers (mf) and their cell bodies can be seen. These cells are predominantly located in the left side of the larva. Small portions of the esophagus (es) are also labeled. (b) Third quartet micromere of the B cell quadrant (3b) of this larva had been injected. In the corresponding fluorescence micrograph (e) a large number of labeled muscle fibers (mf), and their cell bodies, can be seen. These cells are predominantly located in the right side of the larva. Small portions of the esophagus (es) are also labeled. (c) Larva derived from an embryo in which the fourth quartet micromere of the D cell quadrant (4d) had been injected. In the corresponding fluorescence micrograph (f) two loosely organized mesodermal bandlets can be seen; they are located to the left and right sides of the junction between the esophagus and the gut (gt). The left mesodermal bandlet (lmb) is in sharp focus, while the right mesodermal bandlet (rmb) is out of focus in this image. Other scattered labeled mesenchyme cells (ms) are also present. A small portion of the gut endoderm (en) is labeled, which is typical for the mesentoblast cell. (llp) left lappet; (rlp) right lappet. Scale bar equals 50 μ m.

The nemerteans constitute another protostome phylum with embryos that exhibit spiral cleavage. Recent studies revealed interesting differences in their development relative to other spiralian (4, 5). The exact origin of the mesoderm in nemerteans is poorly understood, and the varied literature on the subject lacks consensus (see discussions by 2, 4, 6–8). For instance, some claim that the mesoderm in *Cerebratulus lacteus* arises solely as ectomesoderm (7). On the basis of cell isolation experiments, Hörstadius (8) claimed that some mesoderm in *C. lacteus* was derived from second quartet micromeres, while additional mesoderm was probably derived from subsequent micromere quartets.

To determine the exact origins of the mesoderm in *C. lac-*

teus, we injected individual blastomeres, through the time of fourth quartet formation, with the fluorescent lipophilic dye DiI (3, Molecular Probes Inc., Eugene, OR). Injected cells continued to divide normally, and the injections had no observable effect on development. Embryos were reared at 20–22°C for 48–72 h. The distribution of the fluorescent lineage tracer was determined by examining the resulting pilidium larvae with fluorescence and differential interference contrast microscopy.

Sixty-eight first quartet, 72 second quartet, 37 third quartet, and 40 fourth quartet micromeres as well as 68 fourth quartet macromeres were injected. The larval muscle cells were found to be derived from the third quartet micromeres (*i.e.*, 3a and 3b) of the A and B cell quadrants (Fig. 1a, d and 1b, e, respec-

tively). No other blastomeres contributed to the formation of these mesodermal cells (data not shown). The 3a and 3b micromeres also give rise to small ectodermal clones located in the esophagus (Fig. 1, a, d, and 1b, e). One of the fourth quartet micromeres (4d), derived from the D cell quadrant, generated a pair of loosely organized mesodermal handlets located to the left and right sides of the junction between the esophagus and the gut (see Fig. 1c, f). This cell also gives rise to a population of scattered mesenchyme cells (Fig. 1c, f). The other fourth quartet micromeres, as well as the fourth quartet macromeres, give rise to only gut endoderm (data not shown). These findings indicate that all of the mesoderm contained in the pilidium larva of *C. lacteus* is derived as ectomesoderm from the 3a and 3b micromeres, and as endomesoderm from the 4d micromere. Clearly, the dual origin of the mesoderm in *C. lacteus* is similar to the pattern encountered in other spirilians.

On the basis of ectodermal contributions, the four nemertean cell quadrants in *C. lacteus* were referred to as the left and right ventral (LV, RV) and the right and left dorsal quadrants (RD, LD) (4). It is now clear that these represent respectively, the A, B, C, and D cell quadrants that characterize other spirilians. The data reported here lend further support to the notion that the spiralian developmental program has been highly

conserved. Nevertheless, other differences indicate that significant evolutionary modifications have occurred in the nemertean developmental program (2, 5).

The authors thank the generous community of the Marine Biological Laboratory. J.Q.H. was supported as an MBL Associates Fellow and a Lemann Fellow. M.Q.M. was supported by NSF and as an MBL Spiegel and Davis Fellow.

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Myogenic Differentiation of Sea Urchin Secondary Mesenchyme Cells Is Dependent on an Intact Extracellular Matrix

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The differentiation of muscle precursors in most organisms is dependent upon the expression of muscle-specific regulatory factors belonging to the MyoD family of basic helix-loop-helix transcription factors (1). These molecules activate the entire myogenic program and convert many nonmuscle cells to the muscle lineage (2). Although much is known concerning the targets and expression of these genes, little is known about the activation and regulation of these factors during development. In the sea urchin embryo, myocytes arise from a subset of secondary mesenchyme that delaminates from the invaginating vegetal plate. These sea urchin muscle progenitors, when isolated with associated cells and the extracellular matrix (ECM) and cultured, will differentiate into myocytes *in vitro*, but only if isolated after midgastrulation (3). This is concomitant with the initial expression of the sea urchin MyoD homolog SUM-1 at 36 hours of development (3, 4). Expression of SUM-1 in the intact embryo precedes overt myogenic differentiation of the secondary mesenchyme cells as determined by RNase protection analyses using riboprobes to SUM-1 and myosin heavy chain (MHC) and labeling of cells by indirect immunofluorescence (4, 5). To determine if the ECM plays a role in the activation of SUM-1 and the differentiation of sea urchin secondary mesenchyme cells into muscle, I have removed the ECM from vegetal isolates and examined the ability of cells within

the isolates to express markers of myogenic and endodermal differentiation.

The ECM of the sea urchin embryo contains a heterogeneous mixture of molecules including laminin (6–8); fibronectin (6, 7); heparin sulfate proteoglycans (7); and fibrillar and nonfibrillar collagens (9–11). To remove the ECM, vegetal isolates were treated with collagenases F, H, L, or N, singly, or in combination with pancreatin or with pancreatin alone. Basal laminae visible in control isolates (Fig. 1A) and isolates treated with collagenases alone were not evident in isolates treated with either pancreatin alone (Fig. 1B) or a mixture of pancreatin and each collagenase. Nevertheless, after 36 h of additional culture, the ECM-less isolates express myogenic (MHC) and endodermal (Endo-1; 7) markers at levels comparable to controls (Fig. 1C and D, respectively). These results suggest that the ECM is not required for the differentiation of cells within the isolates. However, it is also possible that cells within the isolates replenish their ECM during culture. To block the deposition of *de novo* synthesized ECM and test whether biogenesis of the ECM might account for differentiation in protease-treated isolates, isolates were cultured in the inhibitor of collagen cross-linking, β -aminopropionitrile (β APN). β APN inhibits gastrulation and differentiation in the intact sea urchin embryo and has a selective effect on the expression of only specific genes (11–13).

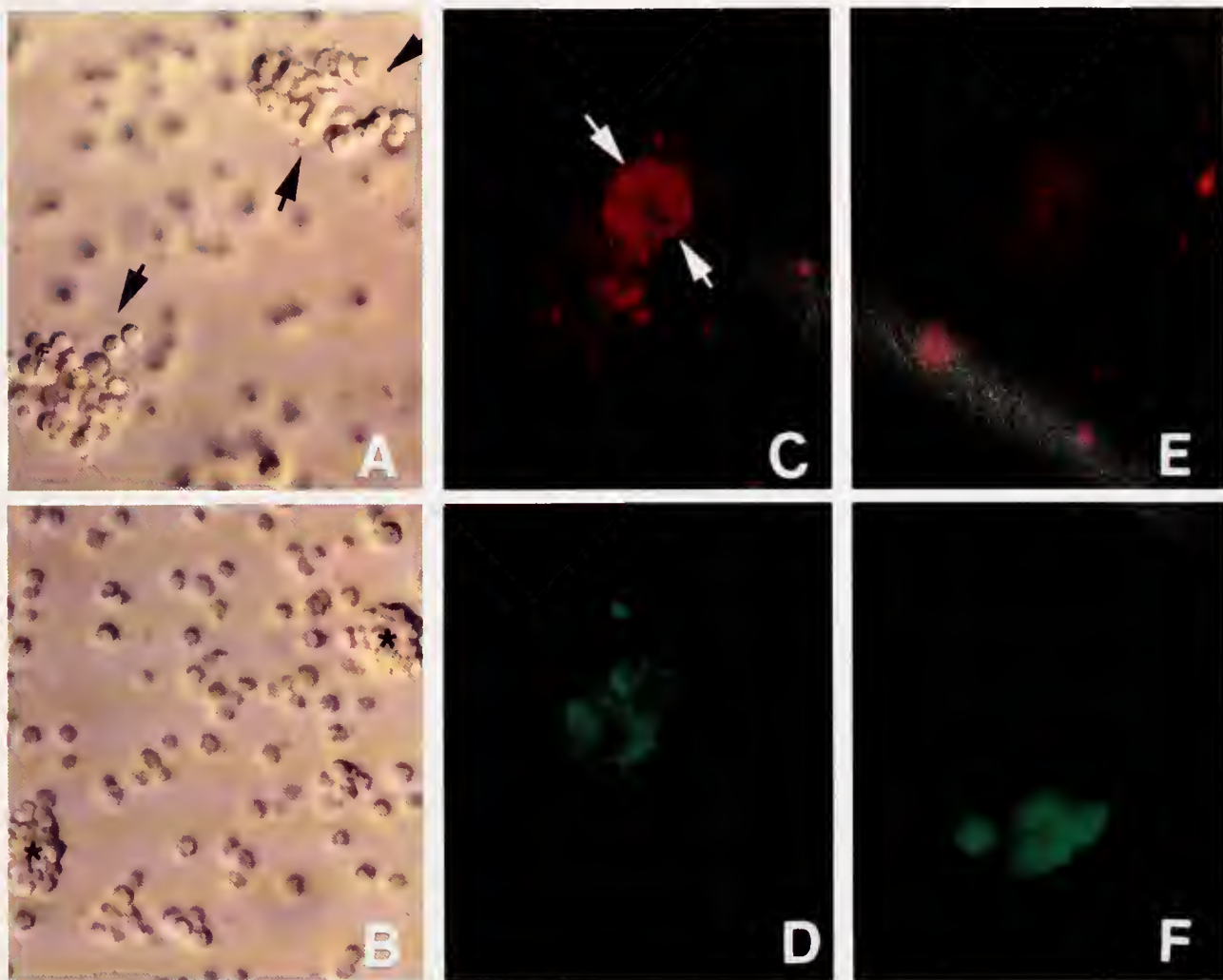


Figure 1. Protease treatment removes the ECM from 36 h vegetal isolates (A and B). When these ECM-less isolates are cultured an additional 36 h and assayed for myogenic and endodermal markers, they express these markers to the same extent as controls (C and D). The expression of myogenic markers is inhibited however, when the ECM-less isolates are cultured in the presence of β APN (E and F).

(A and B) Vegetal isolates were prepared from 36 h *Strongylocentrotus purpuratus* embryos (cultured at 15°C) by sedimentation in a clinical centrifuge at low speed and resuspension in calcium-free seawater (CFSW) three times and in 1 M glycine, 10 mM EDTA, once. This procedure loosens the ectodermal cells from the underlying basal lamina. After repeated sedimentation and washing in CFSW and transfer to filtered seawater (FSW), the vegetal isolates are separated from most of the free ectodermal cells, and the endoderm and mesenchyme cells are retained in a "balloon-like" bag consisting of the basal lamina (arrows in A) and the extracellular matrix that once filled the blastocoel. To remove the ECM, vegetal isolates were treated with either collagenases F, II, L, or N or pancreatin (all from Sigma, St. Louis, MO) at 1 mg/ml in FSW or a combination of pancreatin and collagenase (1 mg/ml each) at 37°C. Throughout incubation in the proteases, isolates were examined on a Zeiss Axiovert microscope equipped with DIC optics to assess the degree of ECM removal. After 30–40 min at 37°C the ECM was no longer visible in pancreatin-treated isolates (B). Control isolates (A) were incubated in FSW for the same time and temperature as the protease-treated isolates.

(C and D) Once the ECM was no longer visible, the isolates were sedimented as above and washed two times with FSW. Protease-treated and control isolates were cultured in FSW with 5 μ g/ml of the antibiotic gentamicin (Sigma, St. Louis, MO) for an additional 36 h. They were then examined for expression of the endodermal and myogenic differentiation markers (Endo-1 and MHC respectively), by indirect immunofluorescence, as follows. Cultures were fixed in methanol at -20°C for 20 min and washed three times with PBS. Isolates were blocked for 30 min in 5% normal goat serum in PBS and then incubated simultaneously in 1:200 dilution of polyclonal anti-MHC (5) and 1:10 dilution of monoclonal anti-Endo-1 (7). MHC-positive cells (arrowheads in C) and Endo-1-positive cells (D) were visualized by using TRITC conjugated goat anti-rabbit secondary antibody at 1:200 dilution and FITC conjugated goat anti-mouse secondary antibody at 1:100 dilution respectively (Cappel, Organon, Teknika), and viewed on a Zeiss Axiovert microscope equipped with epifluorescence optics.

(E and F) To prevent deposition of new ECM, isolates were cultured in the presence of 140 μ g/ml β APN for 36 h and then processed for indirect immunofluorescence as described above. No myogenic differentiation was observed in isolates cultured with β APN when labeled with anti-MHC (E), but endodermal cells expressing Endo-1 were identified in the same isolates (F). Control isolates cultured without β APN expressed both differentiation markers (as in C and D).

Control and protease-treated isolates were cultured with or without β APN respectively. All isolates treated with β APN failed to generate cells that express the myogenic marker MHC, but they contained cells that express the endodermal marker Endo-1 (Fig. 1D and E, respectively). This suggests that the ECM is required for differentiation of secondary mesenchyme cells along the myogenic pathway.

This work was made possible by a Frederik B. Bang Fellowship and an MBL Associates Fellowship. I would like to thank the MBL community for its generous support. This work was also supported in part by NSF award IBN 9506346 to J.M.V. MHC and Endo-1 antibodies were generously provided by Dr. Gary Wessel, Brown University.

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Development and Regeneration of Comb Plates in the Ctenophore *Mnemiopsis leidyi*

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Ctenophores, a group of biradially symmetrical, gelatinous marine organisms, consist of an outer epidermal and an inner gastrodermal layer separated by a mesoglea. Individual muscle, mesenchymal, and nerve cells course through the mesoglea but do not form a definitive tissue layer. Because these acoelomate, planktonic animals lack both internal and external support systems, they are highly susceptible to injury. Accordingly, most larval and adult ctenophores have a high capacity for regenerating missing body regions and all known cell types (1–3). This facility is in contrast to the period of embryonic development in which the embryo fails to regulate (replace missing structures) (3–5). Other workers have found that if defective ctenophore embryos are cultured into the larval period, missing structures are subsequently replaced in a process known as 'post-generation' (3, 6). We have used cell lineage techniques to examine the embryological origin of new structures formed during the larval period within deficient animals in which identified cells were killed during embryogenesis.

One characteristic set of structures displayed by most adult ctenophores are the eight longitudinal rows of comb plates (comb or etene rows) that are used for locomotion. Comb plates arise from polster cells of the outer epidermis and are composed of hundreds of thousands of individual cilia bound together by compartmenting lamellae (7). Ctenophores have a highly stereotyped cleavage pattern, so individual blastomeres

can be identified and their fates followed throughout embryogenesis. Previous cell lineage experiments (4, 8), using chalk particles, revealed that each pair of comb rows is derived from one of the four e_1 micromeres (Fig. 1A). Deletion of e_1 micromeres prevents the formation of etene rows and their associated endodermal canals during the embryonic period, supporting this cell fate assignment (3, 5).

We killed two adjacent adosophaeal e_1 micromeres of the ctenophore *Mnemiopsis leidyi* with glass needles at the 16-cell stage and then injected two other identified blastomeres in the surviving embryo with the fluorescent lineage tracer Dil dissolved in soybean oil according to previously published techniques (9). The blastomeres injected included the two 1E macromeres, two 1M macromeres, and two m_1 micromeres which were all located on the same side as the two deleted e_1 micromeres (see Fig. 1A). In addition, we wanted to see whether the same lineage that makes etene plates during embryogenesis also makes them during 'post-generation', and so we also injected the two surviving e_1 micromeres on the side of the embryo opposite the two deleted e_1 micromeres. This operation generated post-embryonic juveniles (cydippid larvae) that were completely deficient in four of their eight etene rows. Experimental embryos were examined 24–72 h after the operation for the presence of Dil in their newly formed (post-generated) comb plates.

Comb plates that appear during the larval period do not form in the same way that they do during embryogenesis. One to three small comb plates appear in the vicinity of the missing comb rows, but are not organized in distinct rows with uniform

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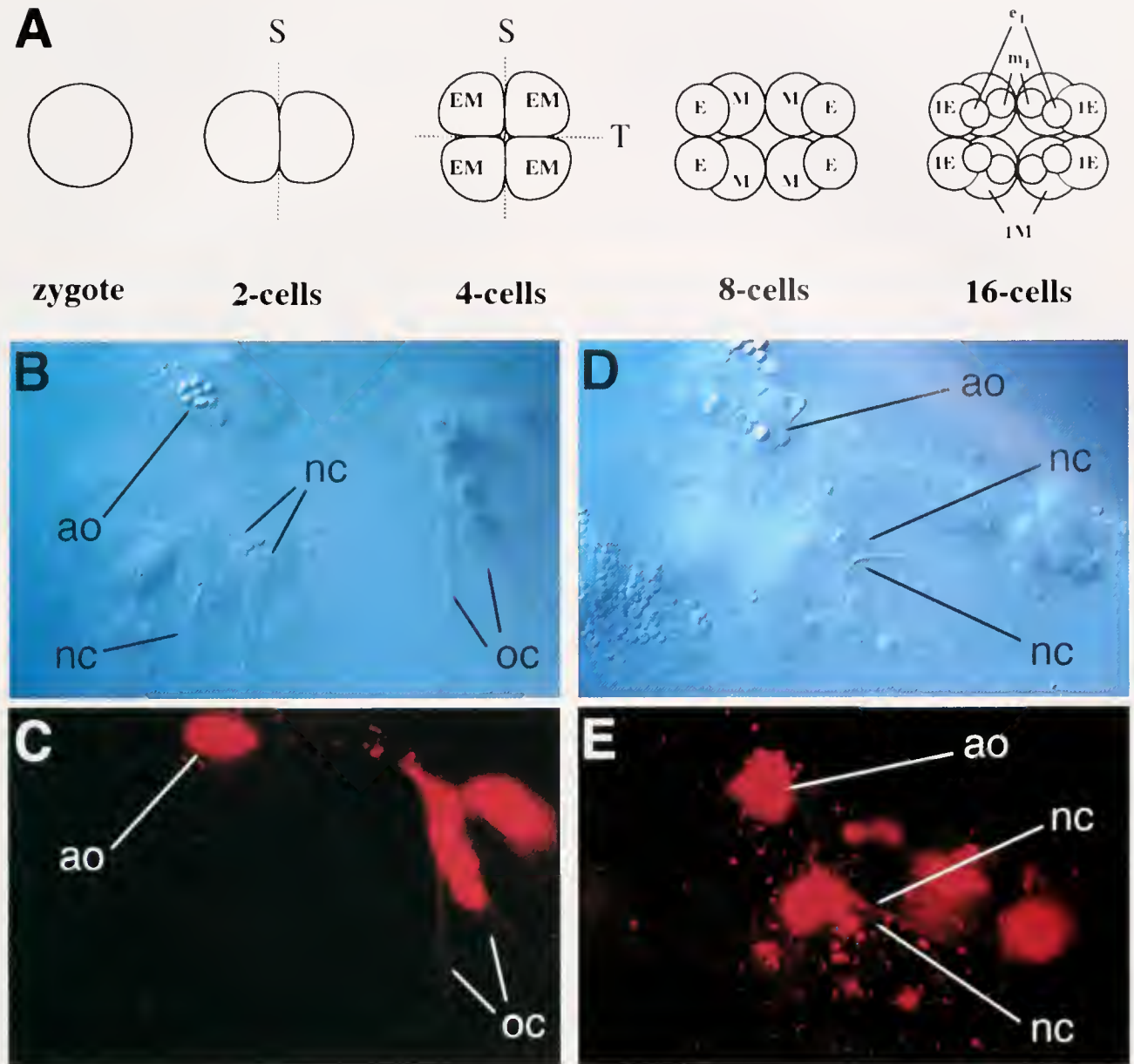


Figure 1. (A) Cell lineage diagram showing the names of individual blastomeres up through the 16-cell stage. First cleavage runs through the adult sagittal plane (S), while the second cleavage plane runs through the tentacular plane (T). At the 8-cell stage the four M and E macromere lineages are established. The 1E and 1M macromeres and e_1 and m_1 micromere lineages are generated at the 16-cell stage. (B) DIC micrograph of a 48-hour-old *Mnemiopsis* cydippid larva in the process of generating new comb plates (nc) following the destruction of two adjacent e_1 micromeres at the 16-cell stage. The apical organ (ao) marks the aboral pole. The original, well organized comb plates (oc) can be seen on the right side of the photo. (C) Fluorescent micrograph of the cydippid larva seen in 1B. The two surviving e_1 micromeres were labeled with DiI at the 16-cell stage following the removal of the opposite two e_1 micromeres. Note the absence of staining in the vicinity of the new comb plates. (D) DIC micrograph of a 36-hour-old cydippid in which 2 adjacent adesophageal e_1 micromeres were destroyed at the 16-cell stage, and the 2 m_1 micromeres on the same side were labeled with DiI. (E) Fluorescent micrograph of the cydippid seen in 1D. Note that both newly formed comb plates are labeled, as is adjacent and subjacent tissue including the apical organ. None of the existing comb plates on the unoperated side of the embryo contain labeled m_1 -derived cells (not shown in this photograph).

polarity and they do not become functionally connected to the apical organ until multiple plates in each row subsequently appear. This pattern closely follows the description of comb plate regeneration in adult animals (2, 3). Our results show that 1E macromeres (12 cases) and 1M macromeres (11 cases) do not

generate cells in new comb plates formed during the larval period (data not shown). Furthermore, the surviving two e_1 micromeres, which make the comb rows on the opposite side of the embryo (Fig. 1B, C), also do not participate in the formation of "post-generated" comb plates (15 cases). But in 18

cases, when the two m_1 micromeres were labeled in the same hemisphere of the embryo as the missing e_1 micromeres, the newly formed comb plates were labeled (Fig. 1D, E). The m_1 micromeres also generate additional structures near the newly formed comb plates, including the apical organ (4, 8), part of the tentacle apparatus, adoesophageal epidermis, and some deep cells subjacent to the newly formed comb plates (Fig. 1D, E). We have not followed the elaboration of newly formed comb rows past 72 h because the Dil fluorescence becomes attenuated over extended periods.

These results suggest that the m_1 lineage, but not that of other blastomeres of the 16-cell-stage *Mnemiopsis* embryo, generates pluripotent stem cell descendants capable of giving rise to novel cell types (comb plates) that they would not normally have generated during embryogenesis. We are investigating an alternative explanation, that previous ctenophore embryonic fate maps may be incorrect, and that m_1 descendants normally contribute to comb plate formation during embryogenesis.

The authors thank the generous community of the Marine

Biological Laboratory. J.Q.H. was supported as an MBL Associates Fellow and a Lemann Fellow. M.Q.M. was supported by NSF and as a Spiegel and Davis Fellow of the MBL.

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Zebrafish Embryo Monitoring of the Aquatic Environment: Dose-Response Synergism Revealed in Combinations of Pollutant Chemical Mixtures

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Extraembryonic membranes, such as the fish chorion, provide a protective barrier between the embryo and the environment. Although the fish chorion excludes many chemical pollutants, some noxious agents can still gain access to the aquatic embryo. Therefore a monitoring system that tests the effect of chemicals directly upon the embryo must be established. Although exposure to a single toxin in the laboratory can determine the concentration at which a pollutant becomes a health or environmental hazard, embryos and adults in nature are not merely affected by a single chemical, but are exposed to mixtures of different pollutants. Our purpose in this study was to test whether the zebrafish embryo could provide an efficient model for the rapid observation of the effects of chemical mixtures on development. Zebrafish are particularly useful for these studies because thousands of transparent fertilized eggs and embryos may be collected daily from a small breeding col-

ony. Depending on the ambient temperature the zebrafish embryo develops and hatches in 2–3 days (1).

In our experiments, embryos were collected each morning, sorted, and staged. At gastrulation [5.25 h post fertilization (hpf)] the embryos were placed for 20 s in a 1.6 μ g/ml solution of trypsin (Sigma), made up in embryo rearing solution (ERS) (Carolina Biological); the embryos were then rinsed three times in ERS. This treatment softened the chorion sufficiently that it could be removed with a pair of forceps. The dechorionated embryos were placed in a petri dish containing ERS, and lined with Parafilm (to prevent sticking). A 250- μ l droplet of the various chemical pollutants was placed in a petri dish (100 mm) lined with Parafilm, and a single embryo was placed in each droplet. After 30 min of exposure, the embryos were removed from the droplets, rinsed three times in ERS, and transferred into Parafilm-lined petri dishes half-filled with ERS. The embryos were examined immediately after removal from the pollutant, and every 24 h until they reached the swim-up stage (96 hpf). Deviations from normal development were video recorded. When the swim-up stage was reached, a final video recording was made and the embryos were killed, fixed, and stained with hematoxylin and eosin for histological examination.

We followed this procedure in exposing embryos to the fol-

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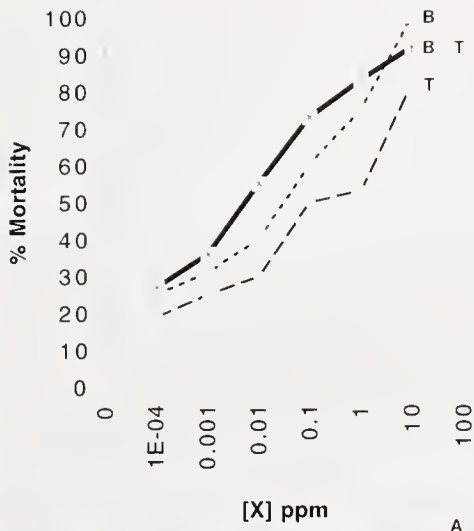


Figure 1 (A) Dose-response relationship of the mortality of dechorionated zebrafish embryos exposed to benzene (B), toluene (T), and a 50/50 mixture of both (B+T). (B) Videoprint of embryo exposed for 30 minutes to a 50/50 mixture of 0.0001 ppm benzene and toluene at the gastrula stage. Photo taken 48 h post fertilization (hpf). Note general stunted development and pericardial edema (arrow) when compared to the control. Magnification bar (0.5 mm) same as control shown in 1C. Forty-eight hpf control animal. Magnification bar = 0.5 mm.

lowing chemicals: benzene, toluene, and a mixture of the two. The concentrations of benzene and toluene in the ERS ranged from 10 parts per million (ppm) to 0.0001 ppm, and the concentrations in mixtures of benzene and toluene were in a similar range. Equal volumes of equal concentrations of benzene and toluene were combined to provide a series of 50–50 mixtures. As the concentration of benzene, toluene, and the mixtures increased, so did the mortality (Fig. 1A). In this preliminary study, the slopes of the curves in Figure 1A are similar. However, at the highest concentration (10 ppm), the slope of the combination of benzene and toluene declines; the significance of this effect will be determined in future experiments.

Dechorionated gastrula-stage embryos were immersed in different toluene concentrations for 30 min, and the lethal concentration that killed 50% of the animals (LC_{50}) was found (0.1 ppm). Benzene, tested in this manner, had an LC_{50} of 0.05 ppm, but the mixture of benzene and toluene had an LC_{50} of about 0.005 ppm. Therefore, the combination of benzene and toluene is acting synergistically; *i.e.*, at intermediate doses, the mixture is more toxic to the embryos than either benzene or toluene alone (Fig. 1A). [TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and a mixture of TCDD and benzene have also been tested, and they display a similar synergism (pers. comm.)].

The retarded growth of treated embryos was immediately evident. Histopathological examination revealed many pycnotic nuclei in the organs of the treated fish when compared to the controls. Although multiple toxin-damaged nests of necrotic cells (often seen in acute toxicities) were lacking, multifocal death of individual cells was common and might represent the occurrence of apoptosis (2). The gills of these animals were poorly developed or even malformed; liver and gut also exhibited minimal differentiation.

Dechorionated zebrafish embryos exposed to benzene, toluene, or the mixtures also displayed specific cardiovascular defects. Histological observation of the heart, atrium and ventricle revealed a smaller tube with smaller and fewer cells than in the control animals. These embryos failed to reach the swim-up stage; their heart rate was greatly reduced, and they exhibited pericardial edema (Fig. 1B). Many of these animals lacked any visible circulation. In previous research, the microinjection of hexachlorobenzene into the perivitelline space of Japanese medaka gastrulae resulted in similar heart defects (3, 4). Eighty-nine percent (89%) of the control zebrafish embryos—untreated except for dechorionation—developed at a normal rate and lacked cardiovascular defects (Fig. 1C).

These preliminary results indicate a need for further study of the effects of various combinations of mixtures of chemicals on embryonic development. The synergistic interaction of benzene and toluene adversely affecting embryonic development is in contrast to the “protective effect” of toluene in adult mice and humans when exposed to mixtures of benzene and toluene (5). This may indicate that during development the toluene-benzene mixtures affect embryos differently than if these fish were exposed to these chemicals as adults. It also raises the question of whether current mammalian transplacental toxicity monitoring is adequate. Additionally, the cardiovascular defects caused by benzene, toluene, and their mixtures are similar to those caused by hexachlorobenzene, dinitrophenol, parathion, carbaryl, tolbutamide, and 2,4,5-trichlorophenoxyacetic acid (3,4,6,7,8,9,10,11). These results indicate that structurally diverse pollutants can have similar adverse effects on cardiovascular development.

The use of dechorionated zebrafish embryos in static exposure assays has provided a sensitive means for uncovering the effects of chemicals and chemical mixtures on early development. The mechanism of action of benzene or toluene and the basis for synergism have yet to be elucidated. The ability to perform genetic analysis combined with the rapidly increasing number of developmental genes that are being uncovered and characterized in the zebrafish make this system suitable for examination of molecular correlates of these defects. This zebra-

fish preparation should also prove useful in monitoring the environment for chemical pollutants.

Supported by grants to M.M. from the Department of Defense, #CBR-93DNA-2; Department of Energy, #DOE-EM-IFG0193E; and the United States Environmental Protection Agency, #CR822766-01-0; and by grants to J.J.S. from the Air Force Office of Scientific Research #(AFOSR)F49620-94-1-0368, and Sea Grant # NA46RG0470, R/P-60.

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Reference: *Biol. Bull.* 191: 294–295. (October, 1996)

Chemically Induced Cardiovascular Defects in Developmental Stages of Vertebrates: Dose-Response and Phenotypic Comparisons in Medaka and Zebrafish Exposed to Aryl Hydrocarbon Receptor Agonists Merle Mizell^{1,3}, John J. Stegeman², Eric Romig³, Roxanna Smolowitz¹, Jennifer Schlezinger², Rajesh Katayani³, Bruce Woodin², and Melissa Mortensen³

The developing vertebrate embryo is a sensitive target for effects of chemical pollutants, and cardiovascular defects are particularly common. The effects of the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have been described in several fish species and include pericardial edema, yolk sac edema, meningeal edema, hemorrhage, and retarded or congested blood flow, often culminating in death (1, 2). The molecular mechanisms of these actions are not known. Zebrafish (*Danio rerio*) increasingly are used in studies of vertebrate development (3), including cardiovascular development (4), and may be useful in revealing the molecular basis of cardiovascular effects of chemicals. We compared the sensitivity to TCDD in zebrafish and in another species used in developmental toxicity studies—the medaka (*Oryzias latipes*); cardiovascular effects of TCDD have already been demonstrated in medaka (5).

Zebrafish and medaka embryos were obtained from colonies at Tulane University. Gastrulae were injected in the perivitelline space (6) with 40 nanoliters of carrier (corn oil) containing concentrations of TCDD to produce doses ranging from 0.004 to 4 pg TCDD per egg. Alternatively, zebrafish gastrulae were dechorionated [5.25 h post fertilization (hpf)] and exposed to various concentrations of TCDD in embryo-rearing solution (7). Developing embryos were examined every 24 h; defects and any mortalities were recorded. Embryos were fixed in formalin at swim-up—about 96 h post fertilization (hpf) (zebrafish) or at hatching (about 2 weeks) (medaka)—and were pro-

cessed for histology or for immunohistochemical detection of cytochrome P4501A (CYP1A) (8) using monoclonal antibody, MAb 1-12-3 (9) against scup CYP1A (10).

Initially we compared lethality with the occurrence of heart defects following microinjection of TCDD. Survival of carrier-injected embryos of either species was 90–100%. Injection of neither 0.004 nor 0.04 pg TCDD per egg significantly altered the survival or hatching success of medaka or zebrafish. At 0.4 pg TCDD per egg, medaka survival declined to 56% at 24 hours, and to 41% by 72 h (Fig. 1A). At 4.0 pg TCDD per egg, survival of medaka was 0 after 48 h. The survival of microinjected zebrafish at 72 h was 86% at 0.4 pg TCDD per egg. The basis for the difference in toxicity at the same egg doses is not yet understood.

Most of the mortality in these studies occurred in the first 24 h. Defects in the heart and circulatory system were observed in both medaka and zebrafish exposed to TCDD, and the defects were seen in the surviving embryos as early as 48 h. These defects included severe pericardial edema, and in the extreme, the normal differentiated two-chambered teleost heart failed to appear; instead, the tubular precursor merely persisted.

Exposure of dechorionated zebrafish embryos to TCDD dissolved in embryo rearing solution (7) resulted in dose-dependent mortality. Zebrafish embryos seemed to be more sensitive to this kind of treatment than to injection. Direct exposure of dechorionated zebrafish embryos may be used to more accurately define the dose and developmental stage at which cardiovascular lesions are initiated.

The molecular mechanism of these effects is not known. A prominent molecular effect of TCDD is the induction of

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1A. Survival of Medaka Embryos Injected With TCDD

Time After Injection	N	24 Hrs	48 Hrs	Heart Defect	72 Hrs	96 Hrs
Concentration	N					
10 ppm	9	1 (11%)	0	0	0	0
1 ppm	32	18 (56%)	13 (41%)	2 (6%)	13 (41%)	13 (41%)
0.01 ppm	24	23 (96%)	23 (96%)	1 (3%)	23 (96%)	23 (96%)
0.0001 ppm	34	34 (100%)	32 (94%)	1 (3%)	32 (94%)	32 (94%)
Control	12	11 (92%)	11 (92%)	0	11 (92%)	11 (92%)

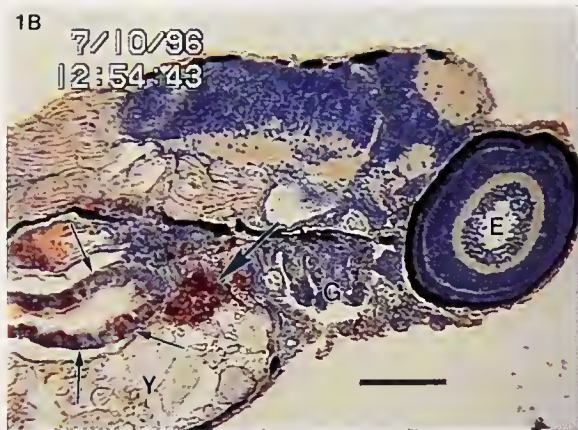


Figure 1. (A) Viability of medaka embryos injected with TCDD at the gastrula stage (24 h post fertilization). Medaka hatch at approximately 14 days. (B) Photomicrograph of 96 h post fertilization (hpf) zebrafish that was dechorionated at the gastrula stage (5.25 hpf) and exposed to 100 µg/ml TCDD for 30 min. Antibody staining demonstrates CYP1A activation in the epithelial cells of the liver (large arrow) and stomach (small arrows). Cells lining the gills, pharynx, and esophagus leading into the stomach are devoid of stain. Y = yolk; G = gills; E = eye. Magnification bar = 0.1 mm.

CYP1A monooxygenases through binding of TCDD to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor (11). Staining of these TCDD-treated embryos with a monoclonal antibody prepared against fish CYP1A (Mab 1-12-3) showed that CYP1A was already present and activated in these 96-h-old zebrafish and newly hatched medaka; expression was seen in multiple organs of both species. At the time of sampling for analysis, this staining was seen prominently in endothelial cells of multiple vascular structures, and in the epithelium of various organs including liver, gut, and kidney, (Fig. 1B).

CYP1A activates or inactivates many foreign compounds; indeed CYP1A and the AhR both might participate in the toxicity of TCDD and related compounds (12). Recent studies (13) have suggested that CYP1A may be causally linked to the effects of TCDD in medaka embryos, possibly through stimulating the release of reactive oxygen species from CYP1A (14). CYP1A also metabolizes retinoids (15) that may be involved in development. The results here substantiate studies that have

demonstrated the expression of CYP1A in developmental stages of medaka (16, 17, and Cantrell *et al.*, pers. comm.) and other embryos exposed to TCDD, and they also show, now, that similar expression occurs in zebrafish embryos.

While the AhR and CYP1A could be involved in toxicity and cardiovascular effects, the proximal mechanisms leading to the effects observed are not known. Moreover, chemicals other than AhR agonists and CYP1A substrates (*e.g.*, toluene, benzene) can also cause cardiovascular defects in medaka and zebrafish (7). Thus, there may be different initial avenues leading to these effects.

This research was supported by grant #CBR-93DNA-2 from the Department of Defense; grant #DOE-EMIFG 0193E from the Department of Energy; and grant #CR822766-01-0 from the United States Environmental Protection Agency to M.M. and a grant from the Air Force AFOSR F49620-94-1-0368 and Sea Grant NA46RG0470, R/P-60 to J.J.S.

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Masking Effects of Sulfate upon *Microciona* Sponge Cell Carbohydrates: A Lectin Histochemical Study

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Carbohydrate residues on many cell types are customarily masked by strong anions such as sulfate (1). These terminal structures are significant in cell function (2); for example, they are involved in immunogenicity, in binding of the underlying sugars to specific antibodies or to lectins, and in secretory phenomena (3, 4). In addition, sulfates protect the underlying sugars from degradation by glycosidases (5). Such sugars can be exposed by chemical or enzymatic methods that remove the anionic structures (6).

Our interest is based upon earlier biochemical findings that adhesive proteoglycans (AP) derived from the marine sponge *Microciona prolifera* are highly sulfated, as are the surfaces of the sponge cells (7). The following histochemical experiments, using lectins with affinities for specific sugars, illustrate masking of cell-surface carbohydrates.

Chemically dissociated sponge cells were pelleted, fixed in formalin, embedded in paraffin, and sectioned at 5 μ m. Lectin histochemistry (8) was carried out with the following horseradish peroxidase-conjugated (HRP) lectins: soybean agglutinin (SBA) with specificity for *N*-acetyl-D-galactosamine (GalNAc), wheat germ agglutinin (WGA) for *N*-acetyl-glucosamine (GlcNAc), peanut agglutinin (PNA) for β -galactose (Gal), *Ulex europaeus* agglutinin (UEA-1) for α -L-fucose (Fuc), concanavalin A (Con A) for α -mannose (man), and *Limulus polyphemus* agglutinin (LPA) for sialic acid (SA). Controls consisted of substitution of phosphate-buffered saline for lectins and use of appropriate sugars to inhibit lectin reactivity.

Desulfation of cell-surface glycoconjugates in sections was carried out with 1% HCl in methanol at 62.5°C for 4 h prior to lectin staining. Methanol control sections were prepared under similar conditions. Immunohistochemical staining with Block 2 monoclonal antibody (MoAb) followed by HRP secondary antibody was used to define sulfated epitope (6, 9).

The results demonstrate that chemical desulfation enhances lectin staining in the case of SBA (Fig. 1a,b), as well as WGA,

Con A, PNA, and UEA-1. However, acid treatment did not enhance LPA staining, suggesting that sialic acid is not cryptic, but is terminal and, therefore, separate from sugar chains with sulfated termini. Block 2 MoAb reacted positively only in sections that were not desulfated by acid.

These findings raise the possibility that *in vivo* enzyme (sulfotransferase)-dependent sulfation may interfere with endogenous lectin binding to specific sugar ligands. Alternatively, sulfate termini may interact with sulfate-binding lectins (10). These biosynthetic events may relate to behavioral phenomena such as tissue regression (11), programmed cell death, and cell regeneration.

The authors thank Dr. Roxanna Smolowitz for help in preparation of the photographs. Financial support to K.M.H. was provided by a Marian and Marcia Armstrong Fellowship and a grant from the Five-College Coastal Marine Program.

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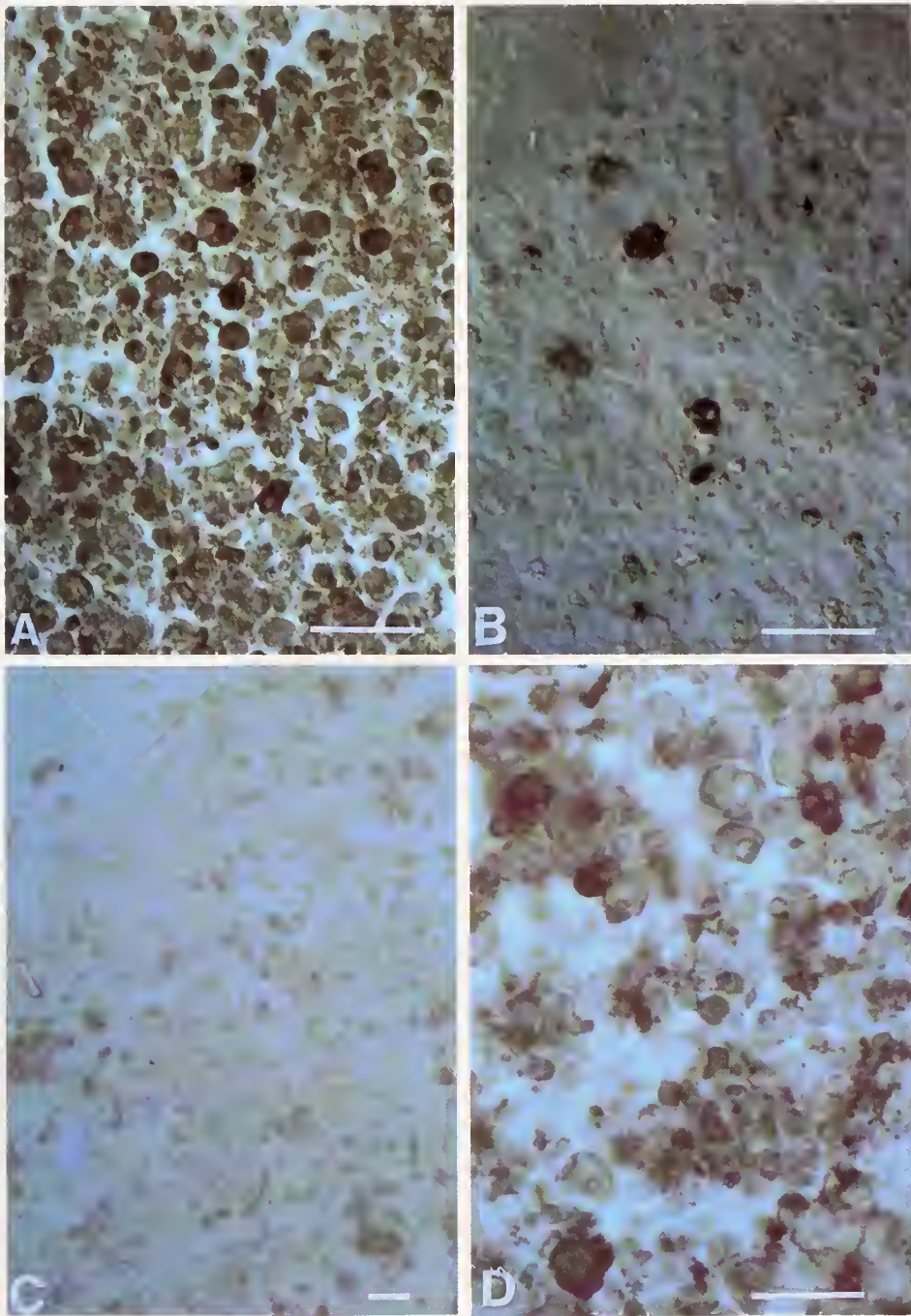


Figure 1. Comparison of HCl-methanol desulfated (A, C) and methanol control (B, D) sections of *Microciconia* cell pellets. A, B. Incubated with peroxidase-conjugated SBA (specific affinity, GalNAc). C, D. Incubated with Block 2 MoAb to sulfated epitope, followed by peroxidase-conjugated secondary antibody. Lectin staining (A, B) and immunostaining (C, D) were visualized by means of diaminobenzidine. Note the reciprocal effect following desulfation between the dark lectin staining of the sugar residue GalNAc (A) and the light immunostaining with the antibody for sulfated epitope (C). Scale bar, 100 μ m.

Reference: *Biol. Bull.* **191**: 298–298, (October, 1996)

The Plasma-Based Cytolytic System of the American Horseshoe Crab, *Limulus polyphemus*: Cooperative Interaction of the Sialic Acid-Binding Lectin Limulin and Thiol Ester-Reacted α_2 -Macroglobulin
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The cytolytic destruction of invading pathogens by proteins of the plasma is an important immune defense strategy of higher animals. The principal protein effector of the plasma-based cytolytic system of *Limulus* is limulin, a sialic acid-binding lectin present in the plasma. In assays with sheep red cells as the model foreign cell, purified limulin is hemolytic at 4–6 nM, and removal of the limulin from plasma eliminates hemolytic activity (1). Hemolysis depends on the sialic acid-binding activity of limulin, because sialylated glycoconjugates, such as fetuin, and the sialic acids N-acetyl neuraminic acid and colominic acid inhibit hemolysis.

The third most abundant protein in *Limulus* plasma (after hemocyanin and C-reactive protein) is *Limulus* α_2 -macroglobulin (LAM), which functions as the principal mediator of protease clearance from the plasma (2). The reaction of LAM with proteases activates a unique internal β -cysteinyl- γ -glutamyl thiol ester bond, generating a reactive glutamyl residue and a free cysteinyl thiol. Activation of the thiol ester initiates a substantial molecular compaction (3), exposing a previously cryptic domain that is recognized by cell surface receptors. Recognition results in the binding, internalization, and degradation of LAM with its cargo of bound protease (2, Ikawi *et al.*, unpub. data). Small primary amines such as methylamine (MA) react with the thiol-esterified glutamine and induce a nearly identical conformational change in LAM, with a concomitant molecular compaction and activation of receptor recognizability. In this report, we identify a new function for LAM: thiol ester-reacted LAM binds to limulin and potentiates its hemolytic activity. Native, unreacted LAM has no effect on the hemolytic activity of limulin.

Limulin (1) and LAM (4) were purified as described previously. Hemolysis was conducted as described in (5). The action of the protease- and MA-reacted forms of LAM is complex: at high concentrations of limulin and LAM, we see a modest depression of hemolysis (6), but at subhemolytic concentrations of limulin, we see a marked potentiation. Potentiation requires a 20–40-fold molar excess of protease- or MA-reacted LAM. This approximates the ratio of these proteins in the plasma: *Limulus* plasma contains 1–5 μ M LAM (7) and about 30–50 nM limulin. Native, unreacted LAM failed to potentiate limulin-mediated hemolysis.

The selective action of reacted forms of LAM on the hemo-

lytic action of limulin is paralleled by the direct interaction of the two proteins. Limulin immobilized on maleic anhydride-activated polystyrene plates (Reacti-Bind-Pierce) bound MA- and protease-reacted forms of LAM 5–20 times better than native, unreacted LAM. Methylamine-reacted, but not native LAM, electrophoretically transferred to nitrocellulose from pore-limit polyacrylamide gels, bound biotinylated limulin. The elution profile of [¹²⁵I]-limulin subjected to column chromatography on Sephacryl S-300 HR resin showed a high molecular mass peak ($M_r > 660$ kDa) if MA-LAM was present. This peak was absent when [¹²⁵I]-limulin was chromatographed alone and was much reduced in the presence of native LAM. This indicates that complex formation between limulin and MA-LAM occurs in solution.

α_2 -Macroglobulin was initially identified in *Limulus* and vertebrates as a protease inhibitor (4) and as the carrier molecule responsible for the clearance of proteases from the blood (2). In mammals, α_2 -macroglobulin also functions as the principal carrier for peptide growth factors in the plasma (8). Here we report a novel function for this protein. Potentiation of limulin-mediated cytotoxicity is restricted to the thiol ester-reacted forms of LAM. Presumably this is important in the activation of foreign cell cytotoxicity in situations of immune defense *in vivo*, because the presence of foreign proteases, and thus of protease-reacted LAM, can be expected to be an indicator of the invasion of the tissue spaces by bacteria and other potential pathogens.

Supported by Grant No. MCB-9218460 from the National Science Foundation.

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Reference: *Biol. Bull.* **191**: 299–300, (October, 1996)

Potentialiation of Limulin-Mediated Cytolysis by Thiol Ester-Reacted Forms of *Limulus* α_2 -Macroglobulin: Identification of Functionally Important Domains

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The hemolytic activity of *Limulus* plasma is mediated by the sialic acid-binding plasma lectin, limulin (1). We have recently demonstrated that the hemolytic activity of purified limulin can be potentiated by thiol ester-reacted *Limulus* α_2 -macroglobulin (LAM)(2). Proteins of the α_2 -macroglobulin family are unique for the presence of an internal thiol ester bond, which in LAM links Cys-999 with Gln-1002 (Ikawi *et al.*, unpub. data). Thiol ester activation hydrolyses this bond, releasing the γ -carbonyl of Gln, which in LAM establishes isopeptide bonds with the ϵ -amino group of Lys-254 (3), and the thiol of Cys. Thiol ester activation is initiated by reaction with proteases and is a key event in protease binding by α_2 -macroglobulin. Activation can also be initiated by small primary amines, such as methylamine. LAM is a large protein with several different functional domains. We have been interested in identifying the domains that are important in its ability to potentiate limulin-mediated hemolysis.

Hemolysis of sheep erythrocytes and purification of limulin and LAM were carried out as described previously (1, 4, 5). The binding of limulin to LAM was quantified with a solid phase assay. Limulin immobilized to microtiter wells was exposed to LAM, and the amount of bound LAM was determined by its subsequent reaction with affinity-purified anti-LAM antibodies and a horseradish peroxidase-conjugated second antibody.

Native, un-reacted LAM lacks free thiols (6). The new thiol exposed by reaction of LAM with proteases or methylamine is necessary for the potentiation of hemolysis because its acylation by treatment with iodoacetamide (0.1 M iodoacetamide in 0.1 M Tris buffer, pH 8.0, 4 h, room temp.) eliminates the potentiating activity of methylamine-activated LAM (Fig. 1). Under the conditions used here, the binding of iodoacetamide was limited to the thiol of Cys-999, because the only [¹⁴C]-labeled tryptic peptides of [¹⁴C]-iodoacetamide-reacted MA-LAM had the peptide sequence of the thiol ester domain, PTG-CGIQNMK (3).

Human and *Limulus* forms of α_2 -macroglobulin share significant sequence identity at key functional domains but show no homology in the stretch delineated by residues 24–105. Instead, this stretch of LAM shows modest sequence homology with human complement factor-8 γ (C8 γ). C8 γ is part of the membrane attack complex generated by the activation of the mammalian complement pathway that inserts in the plasma membrane of targeted foreign cells and that mediates cytolysis. The possibility that this domain of LAM contributes to the potentiation of limulin-mediated hemolysis appears unlikely however, because thiol-reacted, but not native, human α_2 -mac-

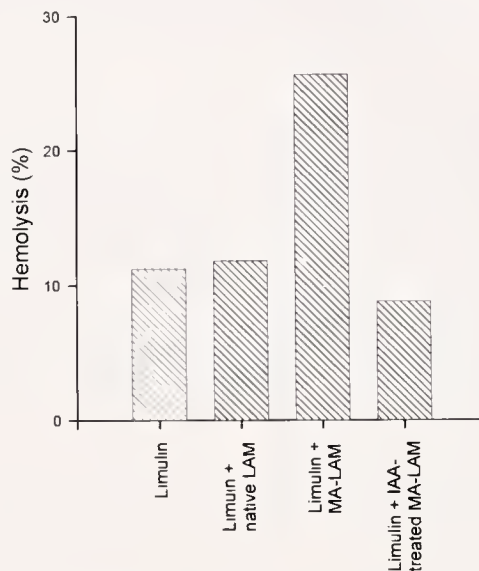


Figure 1. Alkylation of the free thiol of MA-LAM by treatment with iodoacetamide (IAA) abolishes the ability of MA-LAM to potentiate limulin-mediated hemolysis.

roglobulin potentiates limulin-mediated hemolysis with the same efficiency as thiol-reacted LAM. Human α_2 -macroglobulin lacks the C8 γ domain of LAM, demonstrating that this domain is not necessary for hemolysis.

A third domain of interest is the receptor-binding domain, which occupies the carboxyl terminal end of the α_2 -macroglobulins and which is exposed following thiol ester activation. There is significant sequence and functional conservation of this domain between human and *Limulus* α_2 -macroglobulins (thiol-activated *Limulus* α_2 -macroglobulin binds the human receptor). We anticipate utilizing recombinant human receptor-binding domain peptides to investigate the possible role of the receptor-binding domain in the potentiation of hemolysis.

Supported by Grant No. MCB-9218460 from the National Science Foundation.

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Reference: *Biol. Bull.* 191: 300–301. (October, 1996)

Antimicrobial Activity of *Limulus* Blood: Role of C-Reactive Protein (CRP) and *Limulus* Anti-Lipopolysaccharide Factor (LALF)

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The immune defense against invading pathogens is mediated largely by effectors in the blood. *Limulus* blood contains a single type of blood cell, the granular hemocyte (1), and three abundant soluble proteins: hemocyanin (40 mg/ml), the pentraxins (1–2 mg/ml), and α_2 -macroglobulin (0.5 mg/ml) (2). Most of the studies of the immune effectors of *Limulus* describe the role of hemocyte-derived proteins and peptides, which include elements of the blood clotting system and anti-microbial agents, the tachyplesins, the defensins, and LALF (3). These latter agents exhibit anti-microbial activity against gram-nega-

tive and gram-positive bacteria and several species of fungi. This arsenal of proteins helps to maintain a sterile, aseptic, intravascular milieu. In comparison with the well-studied cellular defense systems, relatively little is known of the role of soluble plasma proteins in the immune defense of *Limulus* [for review see (2)].

The subject of this report is the antimicrobial activity of the pentraxins of *Limulus* plasma. This family of proteins in *Limulus* include C-reactive protein (CRP) and limulin, a hemolytic sialic acid-binding lectin (4). The best-studied pentraxins in mammals, C-reactive protein and serum amyloid protein, are acute-phase proteins whose unique functions are still uncertain because of considerable functional overlap with other constituents of the plasma (5). The present report investigates the antimicrobial actions of *Limulus* CRP and compares them to those of LALF, a hemocyte-derived protein. Of particular interest are marine bacteria of the genera *Vibrio* and *Aeromonas*, which are pathogens of chitinous invertebrates including *Limulus* (6). Proteins of the pentraxin family bind to the phosphoryl ethanolamine (PE) and phosphorylcholine (PC) residues of bacterial coat polysaccharides (5). Additionally, limulin recognizes sialic acid residues of glycoconjugates (2). LALF binds to the lipopolysaccharide (LPS) moiety of the cell envelope of gram-negative bacteria (7).

Exposure of *V. alginolyticus* (Fig. 1a) to *Limulus* CRP (200 μ g/ml) arrested cell growth as measured by the kinetic tube assay (see figure caption). The optimal dose of CRP for inhibiting cell growth varied amongst the various *Vibrio* species tested, ranging from 100 to 400 μ g/ml. LALF (200 μ g/ml), limulin (50 μ g/ml) and α_2 -macroglobulin (500 μ g/ml) had no effect on these species, but higher concentrations of LALF (0.5–1 mg/ml) were inhibitory for *V. alginolyticus*. LALF (20–50 μ g/ml) inhibited the growth of *Escherichia coli* (Fig. 1b),

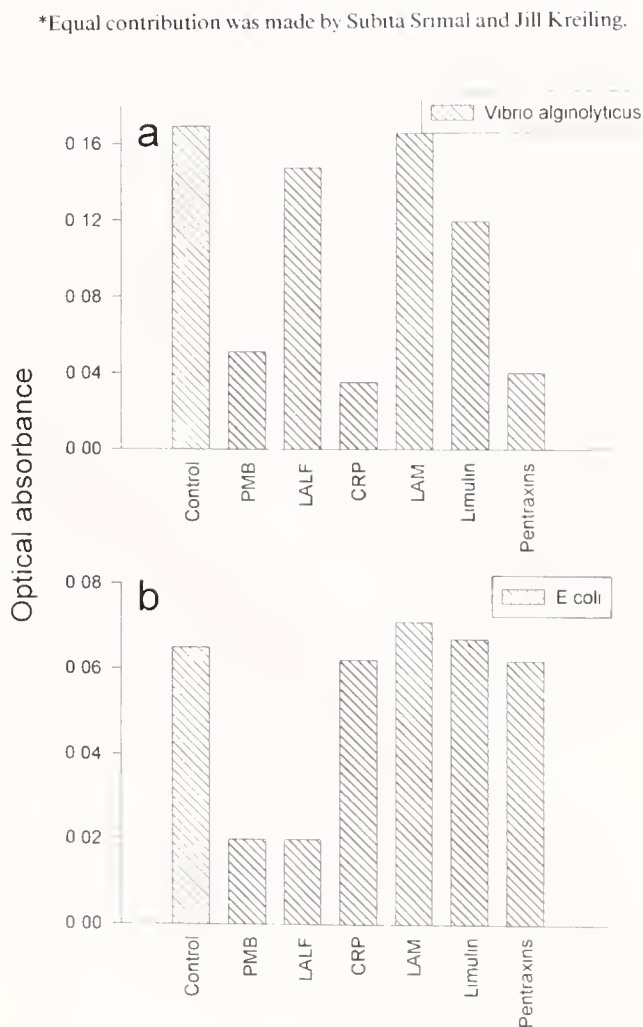


Figure 1. *Vibrio alginolyticus* and *E. coli* cells (2×10^6 cells) were suspended either in seawater or Tris buffered saline (pH 7.5) and incubated at room temp for 2 h with various test samples in a total volume of 1 ml. Aliquots of the treated cells (150 μ l) were transferred into 8 $\times$ 75 mm sterile glass tubes containing 150 μ l of either Marine or LB media (Difco). The tubes were incubated in a kinetic tube reader (ATI) for 4 h and the absorbance was measured with a white light filter every 100 s. Concentration used: PMB (polymyxin B) (250 μ g/ml), LALF (*Limulus* anti-lipopolysaccharide factor) (200 μ g/ml in Fig. 1a and 50 μ g/ml in 1b), CRP (C-reactive protein) (200 μ g/ml), limulin (50 μ g/ml), LAM (*Limulus* α_2 -macroglobulin, 500 μ g/ml), Pentraxins (150 μ g/ml). The approximate concentrations of the plasma proteins in vivo are CRP, 1–2 mg/ml; limulin, 15 μ g/ml; LAM, 0.5 mg/ml.

Staphylococcus aureus, and *Aeromonas* sp. But in contrast to their effects on *V. alginolyticus*, CRP and other *Limulus* plasma proteins had no effect on these species of bacteria.

The treated cells above were examined by differential interference contrast microscopy. CRP caused aggregation of *V. alginolyticus* but not *E. coli*. LALF, in contrast, did not cause aggregation of *E. coli* or *V. alginolyticus* but instead caused lysis of *E. coli*. These observations suggest that the mechanism of action of the two proteins are different, with the CRP appearing to be bacteriostatic and LALF being bacteriolytic (unpub. results). The only reported bacteria pathogenic in *Limulus* belong to the marine genus *Vibrio* (6), and CRP aggregates these cells *in vitro*. Our observation of a bacteriostatic and bacterial agglutinating action of *Limulus* CRP is the first reported function for this abundant plasma protein.

Supported by Grant No. MCB-9218460 from the National Science Foundation.

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Arteries of the Foreflipper of the Harbor Seal (*Phoca vitulina*) as Represented by Contrast Arteriography

J. Donald Schrank, Robert A. Bullis (Laboratory of Aquatic Animal Medicine and Pathology, University of Pennsylvania), Bonnie Jo Smith¹, and Dominique Pennick²

Little information is available on the anatomy of the pinniped forelimb. In particular, there are no descriptions of the vascular system of the foreflipper. Such information is important to pinniped biologists who wish to understand the thermoregulatory physiology of these aquatic mammals and to veterinarians who care for them while in captivity. Because of the lack of availability of protected marine mammals for study, we wished to test the suitability of frozen forelimbs (the animals are frozen when found stranded) for visualizing arterial circulation. Use of frozen specimens has been used successfully in other animals (1).

The forelimbs of three harbor seals (*Phoca vitulina*) were taken at a necropsy session held at the National Marine Fisheries Service in Woods Hole, MA. Each specimen was thawed and examined by gross dissection. Injection of latex was used to accentuate the finer arteries of the foreflipper.

As pinnipeds are believed to have descended from dog-like carnivores (2), standard carnivore anatomic nomenclature was used for all descriptions (3). The initial examinations revealed the absence of major dorsal circulation of the foreflipper. This

finding required confirmation and clarification so radiographic techniques were employed.

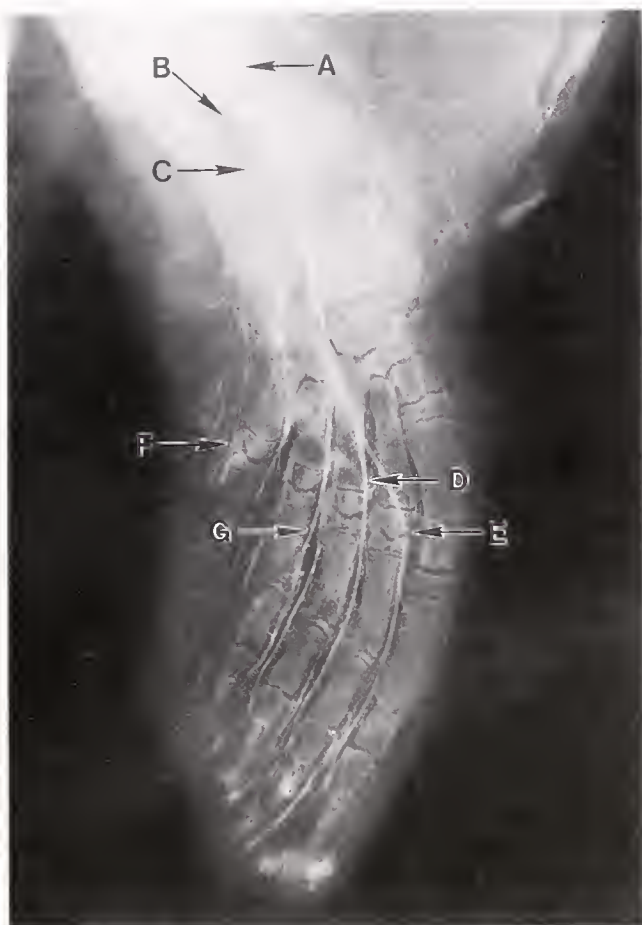
The axillary artery was cannulated with a catheter and the specimen was placed flat, palmar surface up. Sequential radiographs were taken as iohalamate meglumine radiographic contrast material (Conray 60) was injected through the catheter. This procedure continued until no visible increase in intra-arterial contrast material was seen on the radiographs.

Most conspicuously lacking from the harbor seal foreflipper are two major arteries, a superficial brachial artery and a radial artery, and their branches. This results in a reduction of dorsal arterial circulation in the foreflipper. Also missing from the foreflipper is a deep palmar arch. In addition, a variation was found in the blood supply to the abaxial palmar digital artery IV and the axial palmar digital artery V of one seal. These arteries are usually supplied by the common digital artery but in this specimen they were supplied by the caudal interosseus artery (Fig. 1).

The loss of major dorsal circulation in the harbor seal foreflipper is noteworthy because these seals do not regularly haul-out onto iceflows. Other seals (e.g., harp seals) that do haul-out onto iceflows might be expected to have a system that shunts warm blood away from the heat sink of ice. Contrast arteriography of frozen specimens will be a valuable tool for future

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comparative studies of pinniped thermoregulation and anatomy.

Donald Schrank (Kenyon College) was a visiting student at the Boston University Marine Program. We thank Gillian Sanders, Nathalie Ward, Sentiell Rommel, John Nicholas, Winslow Schrank, Elaine Close, and others at the Laboratory of Aquatic Animal Medicine and Pathology for their assistance. Performed under NEFSC, MMPA #917.

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Figure 1. The arteries of the foreflipper of *Phoca vitulina* as revealed by contrast arteriography (iothalamate meglumine, Conray 60). A) median artery; B) common interosseous artery; C) caudal interosseous artery; D) palmar common digital arteries; E) palmar proper digital artery I; F) abaxial palmar digital artery V; G) axial palmar digital artery IV (Kodak Lanex Fine X-ray film, 60 Kv 8 maS 16 ms).

Reference: *Biol. Bull.* 191: 302–303, (October, 1996)

Direct Observation of Contact Digestion in Inside-Out Oriented *Aurelia aurita* Polyps

Dirk Bumann and Alan Kuzirian (Marine Biological Laboratory)

Extracellular digestion in a scyphozoan polyp has been assumed to be mediated by digestive enzymes freely secreted into the small and simple gastric cavity ("luminal digestion") (1). But, histological examination of *Aurelia aurita* polyps, fixed at various time intervals after ingestion of an *Artemia* nauplius, indicated that the prey is in intimate contact with the polyp's gastrodermis throughout digestion, and that digestive enzyme secretion, extracellular digestion, and endocytosis are all highly confined to this region of contact ("contact digestion") (Bumann, Kuzirian, and Storch, in prep.). No digestive enzyme activity was detected elsewhere in the gastric cavity. To confirm this evidence *in vivo*, we compared digestion in *A. aurita* polyps that had been oriented either rightside-out or inside-out. In rightside-out polyps, freely secreted enzymes remain in the gastric cavity, whereas in inside-out oriented polyps, they will be lost to the medium. Hence, digestion should be much less effective in inside-out polyps, if freely secreted enzymes play a significant role. On the other hand, digestion could be func-

tional in polyps of either orientation if contact digestion is indeed the dominant mechanism.

Planulae of *A. aurita* were released by an adult medusa collected in Woods Hole, MA. Polyps were raised from these planulae and cultivated in filtered natural seawater at $21 \pm 1^\circ\text{C}$. Digestion of 7-day-old *Artemia* nauplii in rightside-out oriented polyps was observed with a dissecting scope (six replicates). After capture, the *Artemia* was transported to the base of a septum where it was partially enwrapped by gastrodermal folds. Within the next 2 hours the *Artemia* was digested; the median time to disintegration of the *Artemia*'s eye was 55 min (range 50–65 min). The resulting small food fragments were endocytosed in the same gastrodermal region.

Inside-out oriented preparations were produced with a blunt glass rod and fine forceps, as previously described (2, 3), and were kept in a 100-ml glass dish at $21 \pm 1^\circ\text{C}$. The polyps were wrinkled at first, but relaxed within 24 hours, and survived in the inside-out condition for at least 10 days with no visible sign



Figure 1. Inside-out oriented *Aurelia aurita* polyp, 5 min (left) and 70 min (right) after capture of an *Artemia* nauplius (previously stained for 24 h in 0.01% carmine in seawater to improve contrast). At 70 min, the anterior part of the *Artemia* (white arrow) was wrapped by a septum and gastrodermal folds and has been digested, whereas the tail (black arrow) had no contact with the gastrodermis and is still intact. The bar represents 0.5 mm. g: gastrodermal fold, g: glass rod, s: septum, t: tentacle.

of tissue degradation or reorientation. Inside-out oriented polyps were able to catch 7-day-old *Artemia* nauplii with their tentacles (10 replicates). The tentacles contracted and transferred the prey to the scyphopharyngeal complex. From there, the prey was transported aborally along the nearest septum to the septal base, where it was enwrapped by gastrodermal folds and the basal end of the septum (Fig. 1). Within the next 2 hours the *Artemia* was digested; the median time to disintegration of

the eye was 65 min (range 55–80 min). Digested prey fragments were endocytosed in the same gastrodermal region. Indigestible fragments (mainly parts of the exoskeleton) were transported orally and released to the medium. In summary, the processing of prey by inside-out oriented polyps was very similar as compared to rightside-out oriented polyps.

Enzymes freely secreted by inside-out oriented polyps are diluted by the medium by a factor of about 100,000 as compared to the gastric cavity of normally oriented polyps. The same is true for enzymes that leak from the contact region between the prey and the gastrodermis. The only effective extracellular digestive enzymes are those remaining at the region of contact ("contact digestion"). Our results demonstrating a fully functional digestion by inside-out polyps indicate that enzyme activities at the contact region are sufficient to explain extracellular digestion in normally oriented *A. aurita* polyps. This *in vivo* evidence confirms the previous histological evidence obtained with fixed material.

Wrapping by the septum and folds of the column wall enhances the area of contact and helps to keep the enzymes close to the prey. Similar digestive mechanisms exist in Anthozoa (4, 5), where the prey is wrapped by mesenterial filaments; moreover, the mesenteries of Anthozoa also have dense ciliation, which is involved in particle transport (1). Hence, scyphozoan septa and anthozoan mesenteries have some similar functions despite their proposed independent origin (6).

D.B. thanks the Deutsche Forschungsgemeinschaft for financial support in the form of a postdoctoral fellowship (Bu 971/-1). We thank Gabriela Puls for excellent technical assistance.

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Characterization of Methyl Transferase Activity in the Mandibular Organ of the American Lobster, *Homarus americanus*

Todd Claerhout¹ (Illinois State University), William Bendena², Stephen S. Tobe³, and David W. Borst¹

The crustacean mandibular organ (MO) is the site of synthesis of methyl farnesoate (MF), a sesquiterpene structurally related to insect juvenile hormone (JH). Although the role of MF in crustaceans is not completely clear, several studies suggest that it may have functions that are analogous to those of JH in insects (1). An understanding of its biosynthetic pathway and how this pathway is regulated may be useful in understanding the role of MF. One of the last steps in the synthesis of JH is catalyzed by farnesoic acid *O*-methyltransferase (MeT), which transfers a methyl group from S-adenosylmethionine to either farnesoic acid (FA) or JH acid (2). We describe here a similar enzyme found in the MO of the lobster.

Tissues were removed from lobsters (carapace length 70–80 mm) and homogenized in 10 volumes of buffer A (100 mM phosphate, pH 7.2, 5.0 mM EDTA and 2.5 mM 2-mercaptoethanol). After centrifugation (0.5 h at $450,000 \times g$, 4°C), the cytosolic supernatant was frozen in liquid nitrogen and stored at –80°C. MeT activity was measured as described (2), by incubating equal volumes of the supernatant with buffer B (buffer A plus 0.1% gelatin) supplemented with 0.5 μ Ci S-adenosyl-L-[methyl-³H]methionine (DuPont-NEN, 14.4 Ci/mmol). *Trans,trans* farnesoic acid (FA), *cis,trans* FA, and juvenile hormone III acid (JH acid) were produced from their corresponding methyl esters by saponification and added as indicated. Unless otherwise noted, FA was added at 20 μ M, a concentration that is about 10-fold greater than its K_m (see below). After incubation at room temperature, the reaction was stopped by the addition of methanol. The radiolabeled product was extracted with hexane and counted with a liquid scintillation counter. Analysis of some extracts by HPLC (3) showed that the radiolabeled product eluted in a single peak with the same retention time as MF. The majority of the radioactivity ($91.7\% \pm 1.4$, $n = 4$) injected was recovered in this peak.

The majority (93%) of the MeT activity was found in the high speed supernatant, in agreement with reports that MeT in insects is a cytosolic enzyme (2). MF production was linear with increasing incubation times for up to 45 min. Product formation also increased linearly with increasing amounts of supernatant. Maximum MeT activity was observed at pH 7.2.

Boiling (5 min) of the supernatant eliminated MeT activity. Kinetic analysis of the reaction kinetics revealed that MeT has a K_m for FA of 2.7 ± 0.9 (SE, $n = 3$) μ M. Other related compounds (JH acid, *cis,trans* farnesoic acid) did not appear to be methylated by this enzyme ($n = 4$). MeT activity was not observed in supernatants prepared from other crustacean tissues (green gland, muscle, or hepatopancreas). MO samples incubated without FA produced low levels of radiolabeled MF, consistent with reports that MOs contain FA (4).

Eyestalk ablation (ESA) of lobsters is known to increase both the hemolymph level of MF and the MF content of the MO (5). We therefore investigated the effect of ESA on MeT activity. Five days after eyestalk ablation, the mean hemolymph level of MF in ESA animals was 14.1 ± 4.8 (SE, $n = 3$) ng/ml. This was significantly higher ($P < 0.05$, *t*-test) than the MF level observed in intact animals, which had levels of 2.6 ± 1.0 (SE, $n = 3$) ng/ml. The mean MO weight (0.19 ± 0.03 g) was the same for each group. When the cytosolic supernatants of the MO were analyzed for MeT, they showed significantly greater ($P < 0.001$, *t*-test) activity in ESA animals than in intact animals. MeT activity in MO from ESA animals had 10.0 ± 0.3 (SE, $n = 3$) pmol/100 μ g/30 min while intact animals had 1.5 ± 0.4 pmol/100 μ g/30 min.

These studies demonstrate that MeT activity is present in the lobster MO, and that MeT activity is related to the level of MF in the hemolymph. Thus, MeT activity may be a useful index for estimating the endogenous activity of the MO. In addition, measurement of MeT activity may provide further insight into the regulation of this tissue. Finally, these studies could provide the basis for developing inhibitors of this enzyme. Such compounds could be useful in studying the role of MF in crustacean physiology.

This work was supported by grants from the NSF IBN 9319206 (to D.W.B.).

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Reference: *Biol. Bull.* **191**: 305–305. (October, 1996)

Oxygen Production by Dogfish (*Mustelus canis*) Cornea and Lens Catalase In Situ

Seymour Zigman (University of Rochester) and Nancy S. Rafferty¹

Only catalase produces molecular oxygen from H_2O_2 . Its antioxidant action (*i.e.*, H_2O_2 detoxification) represents the end reaction in the redox enzyme chain. It thus defends cells against oxidation that ultimately leads to cytotoxicity. Catalase activity (*i.e.*, the ability to convert H_2O_2 into O_2 in the epithelial cells of the dogfish lens) is inhibited by UVA irradiation (1, 2), and this causes lens opacification. We have examined the catalase activities of the corneas and lenses of intact dogfish eyes. We have also exposed the anterior segments of dogfish eyes to UVA and studied the effect of this treatment on the catalase activity of the cornea and lens. We have measured catalase activity when $[\text{O}_2]$ is markedly reduced.

Oxygen production from H_2O_2 by the anterior surfaces of corneas and lenses was measured with an O_2 electrode and meter (Microelectrodes, Inc.). Fresh eyes were trimmed and washed with elasmobranch Ringers solutions. "Krazy glue" (cyanoacrylate) was used to attach a plastic cylinder about 1 cm diameter and 1.5 cm in height to the central anterior surface of the cornea, and to the anterior surface of the lens (*i.e.*, with the cornea removed). The cylinder—fixed in this way to the tissue—formed a cup into which was added about 1 ml of Ringers medium. A calibrated O_2 electrode (21% O_2 in H_2O) was inserted into the cup, and the increase in O_2 per min was recorded. Purified beef liver catalase (Sigma) was the standard. The increase in % O_2 /min due to the reaction of H_2O_2 with catalase was determined from a standard curve. This curve was linear between 1.2% O_2 /min and 4% O_2 /min. Table I indicates that the cornea and lens produce O_2 at 1.55 ± 1.01 and $2.80 \pm 1.42\%$ O_2 per min, respectively. 3-amino-triazole (25 mM; a strong catalase inhibitor) totally prevented the catalase activities of lens and cornea.

After 130 J/cm² of UVA irradiation of the eyes, catalase activities were totally inhibited in both the cornea and lens (after cornea removal).

When the O_2 concentration in cups containing 0.6 ml of Ringers was reduced to <2% with N_2 , and 0.3 ml of N_2 -flushed 0.39 mM H_2O_2 was added, corneas and lenses produced O_2 at 0.79% and 1.73% per min, respectively. Solutions with N_2 -flushed H_2O_2 but no tissues, or tissues bathed only with Ringers, did not regain O_2 for at least 3 to 5 min, proving that the O_2 was produced by the cornea and lens catalase. This finding

Table I

Catalase activities of dogfish eye corneas and lenses as determined by O_2 measurements

Treatment	Catalase activities (% O_2 increase per minute)	
	Cornea (cup)	Lens (cup) (cornea removed)
Fresh untreated eyes (39 mM H_2O_2)	$1.55 \pm 1.01^*$	$2.80 \pm 1.42^*$
Eyes incubated for 12 h no UV ($n = 3$)	1.3	2.13
Eyes incubated for 12 h UV exposed (130 J/cm ²) ($n = 2$)	0	0
Eyes incubated 12 h in 25 mM 3-amino- triazole ($n = 1$)	0	0
Fresh untreated eye tissues exposed to 390 μM H_2O_2 in <2% O_2 ($n = 3$)	0.84	1.70

n = number of samples.

* = S.D.

introduces the concept that O_2 could be provided to tissues deprived of O_2 via the breakdown of H_2O_2 by catalase.

Thus, the anterior surfaces of intact corneas and lenses have very active, UVA-sensitive catalase activities. In low oxygen tensions (<2%), catalase in both tissues can produce O_2 . This is very important to the lens, which receives only 5% O_2 from the aqueous humor. The breakdown of metabolic H_2O_2 to produce O_2 may have a respiratory function. When its O_2 tension is substantially reduced (*i.e.*, from about 20%), the cornea also produces O_2 .

Supported by Senior Scientific Investigator Award from Research to Prevent Blindness, Inc., NEI (EY 00459), and Bausch and Lomb, Rochester, NY.

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Developmental Defects in Damselfish (*Abudefduf sordidus*: Pomacentridae) Embryos from Metal Artificial Reefs

Lisa M. Kerr (Boston University Marine Program, Marine Biological Laboratory)

Although damselfish have been observed spawning on metal artificial reefs since the early 1960s (1), this is the first report of developmental abnormalities and increased mortality in fish embryos attributable to the establishment of nests on metal substrate. Here I describe abnormalities in *Abudefduf sordidus* embryos developing on metal substrate, estimate the number of embryos affected in a typical nest, and discuss possible causes for these abnormalities and mortality.

Like all pomacentrids, males of *A. sordidus* establish a nest site by cleaning an area of hard substrate, often biting off encrusting material (2). The males attract females to spawn in the nests and then guard the embryos until they hatch (5 days). *A. sordidus* nests were observed in the field at Johnston Atoll, Central Pacific. Photographs of nests on metal substrates were used to determine the area covered by corrosion that contained dead (opaque) embryos. Corroded or oxidized areas of the metal were easily distinguished by a bright orange precipitate (iron oxide) and by varying degrees of unevenness and deterioration. Non-corroded metal was intact, often covered with microturf algae or fine particles of sediment and organic matter. Samples of embryos were collected directly from areas with corrosion. Due to the patchy nature of the corrosion and the large area of the nests, samples that were collected from non-corroding metal were taken from an area of a nest without corrosion or from a nest on metal that did not contain corroding areas. About 200 embryos, constituting a sample, were removed and examined under a dissecting microscope at 40 $\times$ magnification. Samples of embryos from corroding metal and from concrete were screened for iron content by atomic absorption spectroscopy.

In a survey of 88 *A. sordidus* nests, 40% were on metal, 43% were on a natural substrate (coral and coral rock), and 17% were on some other substrate (wood, plastic, or concrete). No nests were observed on a natural substrate in areas where artificial substrates were available, suggesting that these fish prefer artificial substrates. Dead and abnormal embryos on metal occurred chiefly in actively corroding areas; they were bright orange in color, and were coated with what appeared to be a layer of iron oxide (Fig. 1). In a preliminary analysis of the metal content of the embryos, a sample from corroding metal contained 200 ppm iron, whereas a sample from concrete contained 0.78 ppm iron. The combined incidences of mortalities and abnormalities in nests on corroding metal (mean = 22.7, SD = 14.1), non-corroding metal (mean = 3.5, SD = 1.6), and coral rock (mean = 3.6, SD = 2.9) were significantly different (Kruskal-Wallis $P < 0.001$) ($n = 30$). The list of abnormalities found among early embryos includes enlarged segmentation cavity under the blastodisc, developmental retardation or arrest, and abnormal cell proliferations during gastrulation (Fig. 1B & C). These abnormalities often led to death, which generally occurred before the tail bud stage (24 h). Measurements from photographs of five nests showed that between 0.7 and

15.5% of the nest area was corroding. This corresponds to 3,000 to 65,000 affected embryos in a typical nest of 30 $\times$ 60 cm containing 418,000 embryos.

These lethal abnormalities in *A. sordidus* embryos were correlated with nesting on a corroding metal substrate and could be due to metal toxicity or oxygen deprivation. While several metals have been shown to be toxic to fish embryos (3), and most metal ions readily pass through the chorion (4), the exact composition of the metal substrate is not known at this time. Some metals may also affect embryogenesis indirectly; this includes cadmium, which exerts its toxic effects by altering egg membrane properties (5). These changes in the membrane cause decreased oxygen exchange, which has been shown to cause developmental arrest, retardation, and abnormalities in fish embryos (5). Although there is no evidence at this time for cadmium in the metal substrate, other metals present might be acting similarly. Iron hydroxide particles were found to lodge within the pores of the chorion in fathead minnow embryos, reducing oxygen exchange and overall hatchability (6). Iron oxide particles may also clog the chorion in damselfish embryos, or the corrosion process itself may locally reduce oxygen concentration. Iron concentration was highly associated with one embryo sample in this study, although it is not known whether the iron was on the outside or inside of the embryo. Developmental defects due to parental contamination or other genetic causes are not likely since the abnormalities and resulting mortalities would be distributed randomly throughout the nest and not concentrated in one area. Fungal infections were also not observed. Although increased abnormalities and mortalities were observed in damselfish embryos associated with corroding metal, the specific causes and associated mechanisms will be revealed by future research.

Some damselfishes prefer artificial substrates, and their use often increases reproductive success (7, 8). In this case, the preferential use of metal substrates by *A. sordidus* reduces the number of viable larvae at hatching, which may ultimately affect its overall reproductive success.

Research supported by grants to P. S. Lobel from the U. S. Army Chemical Demilitarization Program, the Office of Naval Research N00014-91-J1591 and N00014-92-J-1969, and the U. S. Army Legacy Resource Management Program, DAMD 17-93-J-3052.

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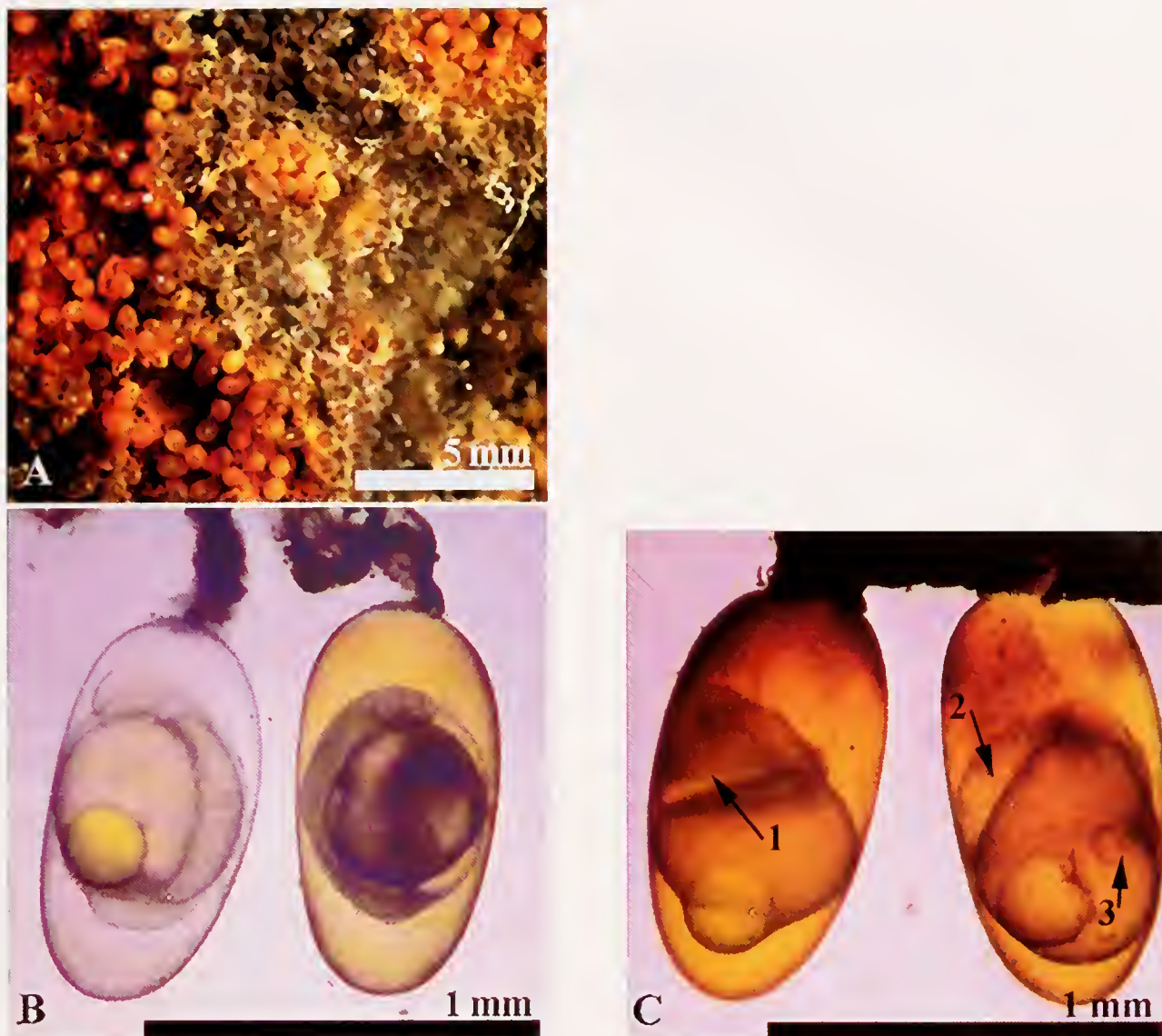


Figure 1. A. Macrophotograph of an *Abudedefduf sordidus* nest on corroding metal. Affected embryos are bright orange. B and C. Photographs of embryos through dissecting microscope. Embryos are from the same nest, are the same age and should be at the same developmental stage as the normal embryo to the left, in B. Normal embryo in B is from non-corroding area of nest. The orange tint on the embryo in B and on both in C is from corrosion products coating the embryo. The right embryo in B illustrates early signs of mortality although development is only slightly retarded. Both embryos in C show developmental retardation. The left embryo has an enlarged segmentation cavity under the blastodisc and shows no sign of beginning gastrulation (1). The right embryo's enlarged segmentation cavity has carried over through early gastrulation (2). Irregular cells are seen along the margin of the migrating blastoderm (3).

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Spawning Sound of the Trunkfish, *Ostracion meleagris* (Ostraciidae)

Phillip S. Lobel (Boston University Marine Program, Marine Biological Laboratory)

Most coral reef fishes are broadcast spawners, releasing gametes that mix in the water column and disperse in ocean currents. Recent studies, with new acoustic technology for recording underwater, revealed that some fishes also produce specific sounds synchronous with gamete release (1, 2). The spotted trunkfish, *Ostracion meleagris*, produces such a spawning sound. It also produces two other types of sounds: an adventitious “bump” when two rival males ram each other’s armored bodies and a short “buzz” in defensive or aggressive contexts.

Audio-video recordings of *O. meleagris* were made at Johnston Atoll, Central Pacific Ocean during October and May 1990, May 1992, April 1994, and May 1996 (see 1, 2 for methods). Acoustic analysis was done using the software Canary (Cornell Univ.) on a Macintosh Powerbook 5300ce. Sounds were digitized at a sampling rate of 11 kHz and a sample size of 16 bits. Sonograms were produced using a 256 pt. FFT and Hamming window with grid resolution of time = 11.61 ms with 50% overlap and frequency resolution = 43.07 Hz. Amplitude was set at logarithmic.

The spawning behavior of *O. meleagris* (3) and other ostraciids (4) has been described (also see 5). Briefly, a male courts a female by displaying and swimming around her and repeatedly nudging at her side and back. They rise together in the water column at least 2 m above the bottom, but frequently higher. Spawning occurs as the pair are side by side, tails together and heads facing slightly apart. Moyer (4) reported that *Lactoria fornasini* (Ostraciidae) produces a spawning sound (“a high-pitched hum”) synchronous with gamete release. Because the hum from within any harem had a consistent “pitch” while those of different harems had noticeable differences in pitch, Moyer reasoned that the males must be producing the sound. This spawning sound is loud enough to be heard underwater by the human ear if ambient ocean conditions are relatively quiet. Unfortunately, Moyer did not record the sounds.

The spawning sound of *O. meleagris* was produced only when a pair were in the mating position and spewing gametes [see (3,4) for photographs]. The sound is of long duration (about 6.3 s) with peak energy at 215 to 270 Hz (Fig. 1a). In the example shown, the spawning sound had its peak frequency at 215 Hz. The amplitude of the sound slowly increases, reaching its maximum at the midpoint, and then decreases to the end. The peak intensity per Hz was –30.4 dB. The fish were about 1.5 to 2 m from the hydrophone during recording. This type of sound pattern is unusual among fishes.

The triggerfish, *Melichthys niger* (Balistidae), actively feeds on newly spawned zygotes of *O. meleagris* and is frequently observed rushing toward a spawning pair. This suggests that *M. niger* is cued to the spawning event by the sound emitted by *O. meleagris*. A spawning sound provides a simple mechanism for synchronizing gamete release and thereby maximizing fertilization, but it may also attract egg predators to dinner.

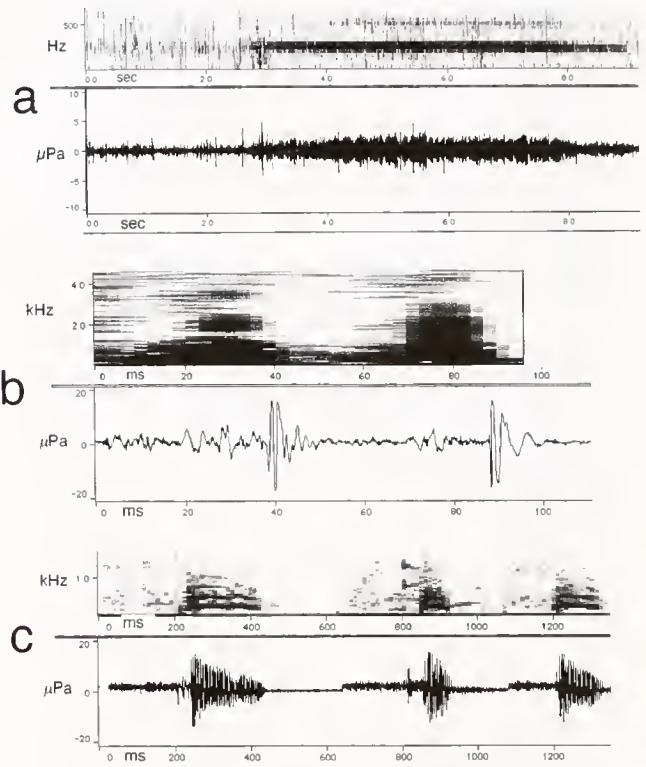


Figure 1. Three sounds produced by *Ostracion meleagris*. (a) Spawning sound, one example. (b) “Bump” of two males jousting; two examples. (c) “Buzz” of defensive or aggressive male; three examples. Each panel shows a sonogram (top) and oscillogram (bottom).

Ostracion meleagris is typically found in groups consisting of a male and several females. In some areas, populations are large, and several males co-occur and compete for mates. Dominant males will actively interfere with and disrupt the courtship and spawning of other males. Competition includes active jousting, with two males facing one another and then ramming together, to produce a “bump” sound (Fig. 1b). The bump is a broad-band sound with peak energy between 140 and 790 Hz and durations ranging from 9.9 to 10.6 ms in the two recordings made. The “bump” had a peak intensity per Hz at –34.5 dB and a peak frequency of 388 Hz. The fish were about 1 m from the hydrophone during recording.

The “buzz” sound of *O. meleagris* was recorded when a hydrophone was placed near a male and he reacted toward it (Fig. 1c); the buzz was also recorded when a male swam rapidly toward a spawning pair to interrupt their mating. The buzz sound had a peak energy between 198 and 535 Hz and durations of 130 to 209 ms ($n = 3$). The buzz had a peak intensity per Hz of

–34.5 dB and a peak frequency of 431 Hz. The fish were within 0.5 m of the hydrophone during recording.

Research support: U.S. Army Chemical Demilitarization Program, Office of Naval Research (Grants N00014-91-J1591 and N00014-92-J1969) and the U.S. Army Legacy Resource Management Program (DAMD17-93-J3052).

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Evolutionary Games That Squids Play: Fighting, Courting, Sneaking, and Mating Behaviors Used for Sexual Selection in *Loligo pealei*

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Each individual's reproductive success is measured in terms of the progeny produced in succeeding generations. The fittest mate possible must be acquired, so that the number and quality of progeny will be maximized. The reproductive behavior of squids is remarkably complex and shows considerable diversity both within and among species (1). Squids use a suite of sensory and effector systems to produce these fast and complex behaviors; visual communication plays a major role, but, as noted below, tactile and perhaps olfactory cues are also important during these intraspecific interactions.

Loligo pealei is biomedically and commercially important, and populations migrate inshore each year to spawn. Yet no detailed ethological study of these squids has ever been con-

ducted. This study is based upon preliminary observations of mating and egg laying in the laboratory (2, 3) and in the sea (4), and its aim is to analyze squid mating systems: (i) on natural spawning congregations recorded with diver-held video as well as from a remotely operated vehicle (ROV); (ii) in outdoor ponds 20 × 20 × 1 m; and (iii) in 2.5 m round tanks in the Marine Resources Center of the MBL. In the latter two circumstances, the mating system could be manipulated by altering the sex ratio, the size of males, and the presence or absence of eggs. Five hours of video were obtained, roughly one hour from underwater and 2 hours each from the ponds (two trials) and the indoor tanks (eight trials). A wide range of behaviors was observed and recorded from the natural spawning ground, as well as in the other two study arrangements.



Figure 1. A. Successful mating by a "sneaker male"(s) as a mating pair is moving towards the egg mass (top center) in 3 m near Woods Hole. The sneaker dashed in very swiftly from about 1 m away and attached itself to the female's arm mass and presumably placed a spermatophore packet there. B. The large paired male was taken by surprise and only reacted 9 s later by trying unsuccessfully to wedge himself between the female and the sneaker(s). The sneaker mating lasted 16 s, after which the female continued during one minute to move towards the egg mass and deposit one egg capsule. The large male guarded her very carefully as she did so, even though he was almost certainly guarding one or more sperm sources besides his own. From a videotape.

All females seem to arrive on the spawning grounds with sperm stored in a receptacle below the mouth (2; pers. obs.); the sperm presumably come from mating in the "head-to-head" position (1, 2, 3) while offshore. Theoretically these animals need not mate again, yet all squids engage in reproductive behavior for much, if not most, of each day, both in the field and the laboratory. When eggs are placed in tank or pond systems, the males approach, investigate the eggs through sight, touch and perhaps olfaction—and then aggressive behavior begins. Large males compete vigorously in agonistic contests with other males for access to females; these contests consist primarily of bright white visual displays that can escalate to mild fin beating. Males and females exchange a variety of body pattern signals as they form temporary pairs that lasted up to two days in the laboratory trials. Large males mate the females in the "male parallel" position (1, 2, 3) and guard them as they approach the egg mass and lay each egg capsule.

"Sneaker" males, which are smaller than the typical large males discussed above, use an alternative tactic. They do not engage large males in agonistic contests; instead they jet forward suddenly onto the arms of a female as the large male and female pair approaches the egg mass. They swiftly deposit spermatophores directly on the egg capsule that is presumably amidst the female's arms (Fig. 1). Thus there are several potential sources of sperm for each egg capsule: stored sperm, the large male, and the sneaker male. To determine the winner (or

winners) of these sperm competition games, some method of paternity assessment must be used, and these are being developed.

Might the female exert some choice over sperm allocation to the more than 100 eggs in each capsule? Many such important evolutionary questions can now be addressed because the range of behaviors in the field and pond is very similar to that in the laboratory. Thus, by adding an egg mass to a group of squids that includes several male-female pairs and a small male, the mating system can be reproduced in the laboratory, and the dynamics of the interactions can be recorded and analyzed.

I thank Arnold Carr, Bill Macy and Bill Grossman for able assistance in the diving operations, Marie-Zoë Bost and John Cigliano for help conducting some of the laboratory trials, and Dave Remsen for help with the figure. A Phantom 300 ROV was kindly provided by the National Underseas Research Center of NOAA (Project UCAP-96-11) at Avery Point, Connecticut, and the outdoor ponds by the Woods Hole Oceanographic Institution.

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Orientation Behavior of the Lobster: Responses to Directional Chemical and Hydrodynamic Stimulation of the Antennules

Carla M. Guenther, Heather A. Miller, Jennifer A. Basil, and Jelle Atema (Boston University Marine Program, Marine Biological Laboratory)

Although turbulent odor plumes contain spatial chemical gradients that could be used for chemotactic orientation (1, 2), the notion that animals use purely chemotactic mechanisms to locate odor sources remains unsubstantiated. Studies of lobsters (*Homarus americanus*) with aesthetasc (*i.e.*, olfactory) sensilla of one antennule removed have shown that bilateral chemical information is important for efficient localization behavior (3). Other studies suggest that lobsters may use certain specific parameters of an odor pulse to identify a spatial gradient leading to the source of the stimulus (1, 4, 5). A direct test of this hypothesis awaits direct stimulation of the antennules with odor pulses of known shape and rate: bilateral stimulus differences leading to turning behavior would provide information on the parameters of odor pulses used for "chemotaxis" (reviews: 6, 7).

We developed a lobster-mounted, four-nozzle odor delivery

system that can jet small, measured volumes of flavored or unflavored seawater toward either antennule, or both, in freely moving, blindfolded animals. Two pulse generator-driven syringe pumps pushed brief pulses of either filtered seawater or a fish juice solution (0.015%) through two small tubing systems (134 cm of 0.76 mm inner diameter; and 200 cm of 1.19 mm diameter). Each of these systems could be switched through three-way valves to deliver pulses toward the left or right antennule, or toward both simultaneously. In these experiments we used a 1-s pulse that delivered 86 (± 38 S.D.) μ l per pulse at a rate of 1 pulse per 15 s. We used 10 food-deprived lobsters (5 male, 5 female; carapace lengths about 75 mm); each animal was used in two trials. A trial consisted of a half-hour acclimation period followed by 30 stimuli distributed in random order among the six conditions: left, right, or bilateral stimuli containing either seawater or fish juice.

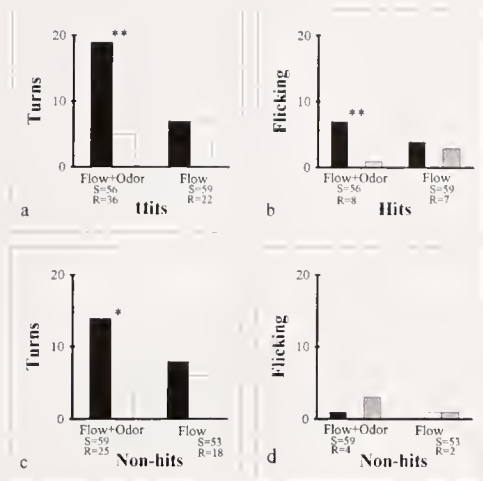


Figure 1. Directional responses of lobsters to unilateral antennule stimulation. Ordinate: number of responses: turns (a, c); number of antennule flick bursts (b, d); Direction of the response: ipsilateral (toward the stimulus, black bars); contralateral (white bars); bilateral (gray bars). Stimuli: flavored (Flow + Odor); unflavored (Flow). (S) number of stimuli; includes "no response." (R) Number of responses; includes non-directional responses not represented in the bar graphs (i.e., stop, move forward, move backward). Hits (a, b): a dyed pulse appears to hit an antennule, determined from video analysis. Non-hits (c, d): dyed pulse appears to miss the antennule (but may still provide flow or some chemical stimulus). Significant ipsilateral responses ($**P < 0.01$, $*P < 0.05$) were turns caused by flavored stimuli (a. Chi square 8.2, c. Chi square 5.5) and flicks caused by flavored hits (b. sign test).

Lobster behavior was videotaped with two cameras: one to record locomotion was fixed above the flow tank (377 cm long, 136 cm wide, 35 cm high, 30 cm water depth, flow rate 1 cm/s), and one was hand-held to record close-up antennule position and flicking, as well as pulse delivery. Both seawater and fish juice were dyed with rhodamine to visualize pulse delivery, thus allowing us to score each pulse as either a "hit" or "non-hit." Whether or not the animal was walking, we scored the following behaviors within 10-s post-stimulus: left or right turn

(30° or more, excluding turns near tank walls), stop, move forward, move backward.

The results show that significant ipsilateral turning occurred not only when fish juice stimuli hit the corresponding antennule (Fig. 1a), but also with fish juice stimuli judged as not hitting the ipsilateral antennule (Fig. 1c). Evidently, our ability to distinguish visually a hit from a non-hit was not shared by the lobsters. Unflavored stimuli did not cause turning toward the stimulated side (Fig. 1a, c). Turns were observed 2–8 s after stimulus release. Flicking was significantly unilateral for flavored hits and never contralateral for flavored and unflavored hits (Fig. 1b); non-hits caused very few flicks (Fig. 1d). Often there was no response, e.g., Figure 1a: 56 stimuli (S) resulted in 36 responses (R), 24 of which were either ipsi- (black bar = 19) or contra- (white bar = 5) lateral turns, and 12 were non-directional.

We conclude that unilateral stimulation of the antennule with food odor pulses can cause ipsilateral turning with 2–8-s delays even if the pulse does not hit the antennule directly, and that unilateral odor pulses hitting the antennule directly can trigger ipsi- and bilateral flicking, but no contralateral flicking. We cannot conclude that odor alone determines directional responses because all of the odor stimuli were embedded in hydrodynamic pulses—a situation common to many natural odor dispersal patterns.

We thank Drs. Frank Grasso, Rainer Voigt, and Daniel Gibson for their input. This study was completed as partial fulfillment of the Major Qualifying Project for the B.S. degrees of C.G. and H.M. at Worcester Polytechnic Institute. Supported by an REU supplement to NSF grant IBN 9222774 to J.A.

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Behavior of Purely Chemotactic Robot Lobster Reveals Different Odor Dispersal Patterns in the Jet Region and the Patch Field of a Turbulent Plume

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In the marine environment, turbulence disperses odor into a chaotic series of discontinuous patches (1, 2). This type of dispersal presents serious challenges to animals, which depend on their sense of olfaction to locate mates and food. The American lobster appears to use its lateral antennules to locate turbulent jets much faster than should be possible if it were computing the spatial concentration gradient from the sum of received patches (3, 4, 5). To investigate the features of a turbulent odor plume that a purely chemotactic animal might use, we use a lobster-sized autonomous robot orienting to a salt plume to simulate the scenario of a lobster hunting for a distant odor source.

The experimental apparatus, the robot, and algorithms are described in detail in our earlier reports (6, 7). The robot, equipped with a pair of conductivity sensors, is placed in a flow-through fresh-water flume (mean flow ~ 1 cm/s) and positioned one meter downstream from an injected jet of salt water (made neutrally buoyant with ethanol). At the start of a trial, the robot is allowed to move freely about the flume, its turns guided by the signals from the two sensors. Two algorithms were used in these experiments. In both algorithms the robot moves forward until a conductivity difference in excess of $20 \mu\text{S}$ across the two sensors triggers the robot to turn in the direction of the stronger signal. Algorithm 2 adds the command to reverse the robot's direction when the signal in both sensors falls below $105 \mu\text{S}$. The speed of the robot, forward and reverse, was 15 cm/s, the speed of a fast-moving lobster. In earlier experiments the sensors were rather widely spaced; in these experiments the separation was 3 cm, matching the lateral antennule spacing of lobsters.

In earlier experiments we observed a change in robot behavior that occurred between 40 and 50 cm from the source; this suggested that the proximal jet (0–50 cm from the source) was an easier orientation task than the distal patch field (>50 cm from the source). To explore this difference, we started the robot at 60 and 100 cm from the source in the experiments reported here. To control for the possibility that a random walk by the robot might lead it to the source, we conducted separated trials in which the sensor signals caused the robot to turn either toward (normal sensors) or away (reversed sensors) from the side of the sensor detecting the higher concentration. For each of the two algorithms, twelve 30-s trials of the robot were run in each of the four conditions: 60 or 100 cm from the source; normal or reversed connections. We evaluated the robot's performance based on the number of "hits" (the number of times

the robot came within 5 cm of the source). We also scored four parameters of the robot trajectories: (1) closest approach to the source, (2) time to closest approach, (3) distance traveled, and (4) the variance of the turns as a measure of path tortuosity. A two-way ANOVA (starting distance by sensor connectivity) was performed on these four extracted parameters.

With reversed sensors, the robot never hit the source, suggesting that the "hits" are not a result of a random walk, and that some feature or features of the odor dispersal pattern are guiding the robot toward the source. With normal sensor configuration, Algorithm 1 did not hit at either distance. But an interaction between starting distance and connectivity showed significantly closer approaches when the robot started 60 cm ($F(1,1,56) = 8.042$ $P < 0.007$) in the normal sensor configuration. Algorithm 2 hit 4 times out of 12 in the normal condition at 60 cm, and once at 100 cm. Closest approach showed significant effects of starting distance and sensor configuration ($F(1,1,56) = 62.3$ $P < 0.001$) without a significant interaction. Taken together these results confirm quantitatively our earlier conclusion that the orientation problem is simpler in the proximal jet than in the patch field.

These hit rates are much lower than those we reported earlier, presumably due to the reduced separation between the sensors in this experiment. The geometry of sensor separation and jet width (narrow near the source) favors wider separations because it is easier for these to span the plume allowing the robot to always keep one sensor in the jet and the other out and track both edges.

This raises the question of why many animals, including the American lobster, seem to have such a narrow sensor separation. Perhaps this reflects the difficulty of navigating the distal patch field. Algorithm 2 scores more hits than Algorithm 1 at the 60 cm starting distance because its back-up instruction allows it to re-enter the plume after exit (whereas Algorithm 1 continues on a straight path away from the plume). Thus Algorithm 2 effectively gives the robot more chances to track the jet edges to the source. This error correction by back-up takes time; thus longer trial durations for Algorithm 2 would likely have increased the hit rate at the 60 cm starting condition. This is not the case for the 100 cm starting condition: closest approach, path tortuosities, distance traveled, and time to closest approach for the normal and reversed sensors are not significantly different and in fact are numerically similar. We interpret the one successful Algorithm 2 hit from the 100 cm starting distance as a chance event, and the hits from the 60 cm starting distance as successful plume edge tracking. Consequently, we conclude that sensor signal difference is adequate for guidance in the proximal jet, but that some other form of information (*i.e.*, more widely separated sensors, a sense of the mean flow or more detailed analysis of the temporal structure

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of odor distribution) is required to navigate the distal patch field to a turbulent jet source.

Supported by NSF Grant BES-9315791 to JA.

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Reference: *Biol. Bull.* **191**: 313–314. (October, 1996)

Obstacles to Flow Produce Distinctive Patterns of Odor Dispersal on a Scale That Could be Detected by Marine Animals

Kevin Dittmer, Frank Grasso, and Jelle Atema (Boston University Marine Program, Marine Biological Laboratory)

Previous laboratory studies of odor dispersal in turbulence have demonstrated the existence of dynamic features in unobstructed jet plumes that could be used by lobsters and other marine animals as cues to the direction and distance of the odor source (1, 2). We undertook the present study to test whether simple obstacles introduced into the flow would affect the informational value of these dynamic features for an orienting animal.

We studied two plumes in a fresh-water flow-through flume [for experimental apparatus see: (2)]. The plumes were formed by injecting salt water (with ethanol added for neutral buoyancy) at 100 ml/min (plume 1) and 150 ml/min (plume 2) into the mean fresh-water flow (~ 1 cm/s). Measurements with a stationary conductivity probe (sampling resolution ~ 1 mm) were taken from an established plume, and the conductivity fluctuations produced by the passing salt plume were recorded (sampling rate, 40 Hz). Recordings were made at two sites, 75 and 100 cm downstream from the source. Differences in dynamic concentration parameters at the two sites would indicate a spatial gradient where a pure concentration gradient does not exist on a time scale suitable for potential use in chemotaxis. Additionally, measurements were recorded from plumes established with a cylinder (6 cm diameter) or a plate (6 cm wide oriented perpendicular to the mean flow) and placed 50 cm downstream from the source along the mid-line. To control for the confounding effects of the sequence in which conditions were examined, we made three independent replicate recordings, of four min each, for each condition. The order of the recordings (distance, obstacle, and repetition) was randomized. From each recording, we extracted as dependent variables peak height (PH) and peak onset slope (PS), reliable gradient cues in earlier studies (1, 2). A two-way ANOVA [obstacle by distance, $F(1,2,2,1961)$ plume 1, $F(1,1,1,1551)$ plume 2] was performed on each peak parameter from each plume.

The results confirm our earlier findings that, when unobstructed, mean PH and PS are significantly lower at 100 cm (unshaded bars) than at 75 cm (shaded bars) in plume 2 ($P < 0.01$ PH and PS), but not plume 1 (see Fig. 1). The lack of a gradient in PH and PS in plume 1 has now been seen repeatedly and cannot currently be explained. In plume 1 the introduction

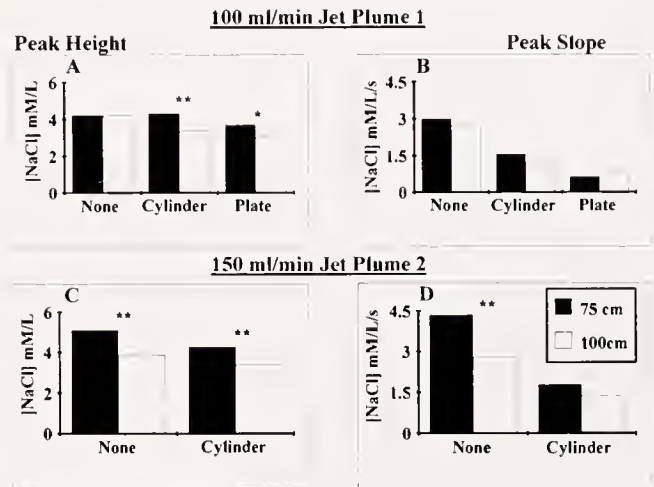


Figure 1. (A) Plume 1 (100 ml/min injection rate) mean peak height for 75 cm (shaded) and 100 cm (unshaded) sites. Significant differences between sites appear with the addition of either object. (B) Mean PS for plume 1 shows no significant effect between sites. (C) Plume 2 (150 ml/min injection rate) graph of mean PH. Significant site differences are retained after the addition of the cylinder. (D) Mean PS for plume 2. The cylinder causes the highly significant site differences in PS 75 to 100 cm to decrease to marginal significance ($P < .059$). Significance levels are indicated by asterisks: * $P < 0.05$, or ** $P < 0.01$.

of an obstacle induced a decrease in PH with distance ($P < 0.01$ cylinder; $P < 0.05$ plate). In plume 2, the introduction of a cylinder did not abolish the significant decrease in PH with distance, and preserved the PS difference at a marginally significant level ($P < 0.059$).

In an unobstructed plume where PH and PS decrease with distance, PH and PS retain their usefulness to orienting animals as spatial gradient cues to an odor source when a single obstacle is introduced. Further, in an unobstructed plume (plume 1) where such a gradient did not exist, introducing an obstacle induced a significant difference in PH. We conclude that under our experimental conditions animals would be able to use PH

and PS as cues to the source in the presence of simple intervening obstacles to flow.

Supported by REU Supplement for K.D. to NSF BES-9315719 to J.A.

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Filament Tracking and Casting in American Elvers (*Anguilla rostrata*) S. J. Oliver, F. W. Grasso, and Jelle Atema (Boston University Marine Program, Marine Biological Laboratory)

American eels (*Anguilla rostrata*) are a catadromous species that uses olfaction to locate food and suitable freshwater habitats (1). During an experiment to determine how anguilliform locomotion (propulsion by passing lateral waves down the entire body length (2)) of elvers affects chemo-orientation, we observed elvers casting and filament tracking, two behaviors that are previously unreported in this genus. Here we report these observations and suggest a possible quantitative difference between the behaviors.

Migrating elvers are normally positively rheotactic. Casting is a zigzag swimming pattern perpendicular to the mean flow that enhances the likelihood of reentering the odor plume but does not result in significant upstream progress (3). Filament tracking (as defined by the authors) is following the odor filaments, ribbonlike odor patches that are longer in one spatial dimension than in the other two, towards the source. This is different from pure rheotaxis in that the path includes movements along filaments that are sometimes oblique or orthogonal to the direction of mean flow.

Elvers were kept in flow-through seawater tanks and fed a diet of brine shrimp (*Artemia* spp.). The behavior was observed in a 300 × 90 cm flow-through-seawater flume (mean flow 5 cm/s) with a water depth of 12 cm. The observation arena within the flume (90 × 150 cm) was monitored by overhead video. A stimulus solution was injected parallel to the mean flow through a glass capillary tube (ID 1 mm) to generate a filamentous plume. The plume consisted of either a solution of brine shrimp supernatant, rhodamine dye (for visualization), and seawater or dye and seawater. The stimulus concentration of the odor-bearing plume at the source (4.7 parts per thousand) was comparable in magnitude to what the eels are likely to encounter in the field. The eels were released from the chamber (singly or in groups, depending on the trial) directly into the plume. A trial for a particular eel ended when one of three events occurred: (1) the eel located the source (a 'hit'), (2) the eel left the observation arena, or (3) five minutes elapsed. Paths of the eels were digitized at 2 Hz using NIH ColorImage 1.51 software. Subsequent analyses of three representative paths demonstrating rheotaxis, casting, and filament tracking were digitized at 30 Hz.

We conducted a total of 106 trials (96 odor plume, 10 dye

plume) with each eel being run in only one trial (Fig. 1A). Eels usually exited the plume upon release, and the subsequent encounters with the plume were used for analysis. Six of the ten eels run in dye-only trials encountered the plume but showed no response despite contacting individual filaments of dye. In odor-plume trials, 15 eels left the plume boundary and swam upstream (usually parallel to the flume walls) out of the arena. Twenty-five eels encountered the plume but showed no reactions as they continued upstream. Nineteen eels responded to the plume by stopping briefly, but then continued upstream with no further responses. Thirty-seven eels encountering the plume showed behaviors that appear chemotactic (e.g., casting and filament tracking, slower swimming speeds). Twenty-five of these eels followed the plume with casting (2 eels), filament tracking (7 eels), or both (16 eels) but failed to find the source. Twelve of the thirty-seven 'hit' the source, displaying filament tracking (2 eels) or both casting and filament tracking (7 eels).

We calculated the instantaneous swimming speeds and relative turning angles for three representative paths that were digitized at 30 Hz. This is high enough to capture the anguilliform motion. We used a 500-ms averaging window for both swimming speed and turning angle to smooth the effect of anguilliform locomotion. Figure 1B plots the turning angle and speed of a dye-plume eel (squares) and two odor-plume eels that displayed filament tracking (circles) or filament tracking and casting (crosses). The two odor-plume eels moved slower (mean speed 12 cm/s) than the dye-plume eel (mean speed 26 cm/s) and had larger turning angles (mean of 8 vs. 3.5 degrees respectively). Eels in the odor plume moved slower when filament tracking (mean speed 11 cm/s) than when casting (15 cm/s). The casting eel had larger instantaneous turning angles when casting than did either odor-plume eel when filament tracking (mean of 5 vs. 10 degrees). The three clusters on the scatterplot suggest a quantitative difference between rheotaxis (high speeds, small turning angles), filament tracking (low speeds, moderate turning angles), and casting (moderate speeds, high turning angles) that should be useful for characterizing chemo-orientation behaviors of this species in future experiments.

Our observations of filament tracking suggest that eels are using at least two, possibly quantitatively distinguishable, alternative orientation behaviors as part of their chemotaxis rep-

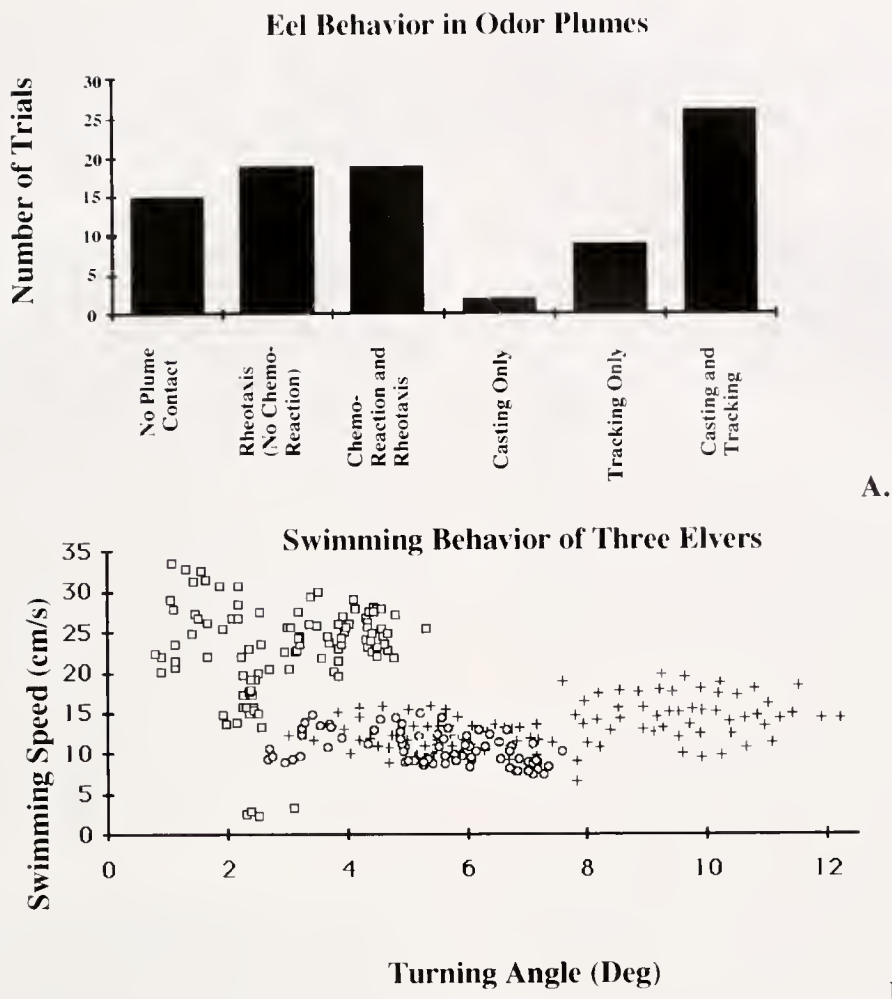


Figure 1. (A) Histogram of the categories of eel behavior observed in the trials. There are three categories of rheotaxis (no plume contact, no response to plume, and response to the odor plume, followed by rheotaxis), and three of chemo-orientation (casting only, tracking only, and casting and tracking). The categories are exclusive, so casting and tracking does not include casting only and tracking only. Dye-plume trials are not included, so the sum of all histograms is 96 trials. (B) Scatterplot of swimming speed versus turning angle for the three paths digitized at 30 Hz. The points represent frame-by-frame positions of the dye-plume eel (squares) and two odor-plume eels that displayed filament tracking (circles) or filament tracking and casting (crosses). The points for the dye-plume eel and the filament-tracking eel form separate clusters. The points for the filament-tracking-and-casting eel fall into two clusters, one separate from the other two eels, and one overlapping the cluster of the filament-tracking eel. The filament-tracking behavior of the tracking-and-casting eel overlaps that of the filament-tracking eel, while casting behavior forms a third cluster. Note that although the scatterplot is for three eels, some combination of casting and filament-tracking occurred in 37 eels (38%), with 12 of them (12.5%) finding the source.

ertoire. The nature of the response to the odor plume suggests two possible orientation strategies; either the eels are using chemical cues to override the rheotactic cues temporarily; or they are using chemical cues to indicate which local currents (which may be oblique, perpendicular, or counter to the mean flow) to follow. In either case, the implication is that some form of chemosensory input is modifying the normal rheotactic behavior of these elvers.

This research was supported by the Boston University Marine Program. We thank Clair Balint, Rainer Voigt, Jenny Basil, and Phil Lobel for sharing their resources and advice. Thanks to Peter Behr and Kevin Dittmer for technical assis-

tance. Jean Frasier and Paul Parsons provided the elvers and the suggestions on how to maintain them. Diana Ma introduced us to the chemotactic behavior of eels.

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New Evidence for Bacterial Diversity in the Accessory Nidamental Gland of the Squid (*Loligo pealei*)

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In sexually mature female cephalopods the accessory nidamental gland (ANG) harbors a dense bacterial community (1, 2). The ANG, which is colorless in immature animals, becomes red-orange in reproductively mature animals; the color has been attributed to pigments of symbiotic bacteria (3). Only a few bacterial types have been isolated from the glands and these only superficially characterized; thus, their identity remains unclear. Recent examination of the genetic composition of this bacterial community by molecular approaches revealed an rDNA sequence that suggested the presence of phototrophic nonsulfur bacteria in the gland. By combining standard cultivation with modern molecular techniques, we began characterization of the bacterial population of the ANG in the common squid (*Loligo pealei*). Results of this study should lead to hypotheses regarding the putative symbiotic role of the bacterial community in the ANG.

Live, mature female *Loligo pealei* ($n = 6$) were obtained from the Marine Resources Center at the Marine Biological Laboratory. The ANGs were removed under aseptic conditions by dissection and washed in filtered, autoclaved seawater. Sterile capillary pipettes were used to collect fluid from different internal areas of the glands. A sterile homogenator was also used to homogenize other glands. The fluid, as well as the gland homogenate, was streaked onto solid media for direct isolations. Liquid enrichment media were inoculated by serial dilution (from 10^{-2} to 10^{-8}). The cultures were incubated under aerobic, anaerobic, and microaerophilic conditions in either light or dark. The incubation temperature was 15°C except for the specific vibrio enrichments, which were incubated at 37°C for 7 and 18 h, and the phototroph enrichments, which were incubated at room temperature. Media were chosen for phototrophic, pigment-producing, luminescent, and other heterotrophic marine bacteria. Phenotypic analyses included Gram stain, colonial and cellular morphology, cell motility, flagella staining, and oxidase and catalase activities.

Bacterial DNA was extracted directly from homogenized ANG as well as from some isolated and cultivated bacteria. Universal prokaryote primers were used to amplify rDNA genes for all samples, and the PCR products were first analyzed by electrophoresis in 1% agarose gel. Purified PCR product (rDNA) from ANG was then inserted into a commercial plasmid vector (pCNR) and transformed into competent cells of *Escherichia coli* by using a commercial kit (5, 3 Prime, Inc., Colorado). Clones were sequenced using the SequiTherm Long Reader Cycle sequencing kit for Li-cor fluorescent sequencing (Epicenter Technologies). For sequencing, 100 to 400 ng of template DNA was used with the 536 reverse 16S rRNA

primer. Sequences obtained were compared to the non-redundant nucleotide database at the National Center for Biotechnology Information by using their World Wide Web site, and the BLAST (Basic Local Alignment Search Tool) algorithm. Phylogenetic analyses were done according to Paster *et al.* (4).

Twenty phenotypically different colonies were isolated from the ANG. Many of these isolates closely resembled those described in previous studies (5, 6). Three isolates were putatively identified by rRNA sequence analysis. A 1350-bp rDNA sequence placed one isolate within the genus *Alteromonas*, having 96% of its sequence identical with *Alteromonas citrea*. This bacterium, which was isolated from all gland samples, was orange pink pigmented and grew both aerobically and anaerobically on a variety of media, including marine minimal agar. The flagella stain revealed a single polar flagellum, as is characteristic of *Alteromonas* spp. Another isolate yielded a 450-bp DNA sequence identical to that of *Aeromonas allosaccharophila*. This isolate grew on a variety of media under aerobic conditions and was apparently capable of reducing S⁰ during anaerobic growth. Finally, one isolate was identified as a member of the genus *Vibrio*, with 96% identity to *Vibrio anguillarum*.

Seven rDNA sequences (400–450 bp) obtained by PCR and cloning from a freshly homogenized ANG were analyzed phylogenetically. All these clones were closely related to a group of alpha proteobacteria that includes the genera *Roseobacter*, *Rhodovulum*, *Rhodobacter*, and *Paracoccus*. Five clones were identical and most closely related to the genus *Roseobacter*, with 93% identity to *Roseobacter denitrificans*. The other two clones were identical to one obtained by one of us (C.-C. Chien) during a 1995 course at MBL as having 88% identity to *Rhodovulum euryhalinum*.

An actual symbiotic role for bacteria in the ANG has not been demonstrated. Recovery of both *Vibrio*-like and pigment-producing strains also in previous studies (5, 6) suggests a specific association between these bacteria and the ANG in sexually mature female squids. In this study one of the *Vibrio*-like prevalent bacteria of the ANG community was orange pink pigmented and was identified by rDNA analysis as a member of the genus *Alteromonas*. For this genus, protection of eggs from fungal attack has been inferred (7, 8, 9). Because the ANG is a reproductive organ and is located immediately adjacent to the egg-producing nidamental glands (9), the antifungal activities of *Alteromonas* may suggest a mutualistic role for ANG bacteria in the cephalopod's life cycle. Also, because only sexually mature female squids have pigmented ANGs, the production of pigments as well as allelochemicals may be an important adaptation allowing microbes to affect their host and competitors. Regarding the other *Vibrio*-like isolates, we cannot exclude the possibility of environmental contaminations. In fact, the isolates identified as *Vibrio anguillarum* and *Aeromonas allosaccharophila* are often found as pathogens in marine ani-

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mals, suggesting that these organisms could simply be parasitic (10, 11). Further work will focus on culturing, identifying, and characterizing the organisms responsible for the DNA sequences found in the bacterial community of the ANG of *Loligo pealei*, and will seek to detect and identify other members of the community.

This combination of traditional culture approaches with molecular analyses provides evidence of a much more diverse bacterial community within the ANG of *Loligo pealei* than had been observed previously. This molecular approach indicates the presence of different and unexpected bacterial types. Particularly novel is the possibility that phototrophic bacteria may play a role or roles in the ANG. We note that although the molecular data presented are not complete, they permit the formulation of sound hypotheses for further investigation, in combination with the microbiological and physiological techniques essential to develop a reasonable understanding of the composition and function of the ANG bacterial community.

We thank members of the MBL Microbial Diversity Course staff and faculty, in particular the Course Directors, E. Leadbetter and A. Salyers, for logistical support and valuable discussions on the project, B. Paster for assistance with molecular analyses, P. Dunlap for discussions on ANG research, and J.

Armitage for discussions on phototrophic bacteria. The course was supported by the Office of Naval Research, the Department of Energy, and the Foundation for Microbiology.

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Surface-Dwelling Bacteria on the Eggs of *Crangon septemspinosa* Restrict Infection by the Fungus *Lagenidium myophilum* In Vitro

Kevin J. Barry and Norman R. Wainwright (Marine Biological Laboratory)

The sand shrimp *Crangon septemspinosa* is a common inhabitant of Atlantic east coast estuaries from the Gulf of St. Lawrence to eastern Florida (1). Like many crustaceans of the order Decapoda, the females incubate their eggs on pleopods on the abdomen until hatching. During the brooding period, the eggs are exposed to the aquatic environment and microbial pathogens for extended duration. Considering the limited egg-preening capabilities by the female, the otherwise unprotected eggs seem to survive microbial encroachment. Eggs of *Homarus americanus*, the American lobster, and *Palaemon macrodactylus*, an estuarine shrimp, are remarkably resistant to infection by the fungus *Lagenidium callinectes*, a known crustacean pathogen (2, 3). In those studies, the eggs were found to host an epibiotic bacterium that produced a molecule toxic to *L. callinectes*. The present study examines eggs of *C. septemspinosa* in an attempt to elucidate whether these eggs are also protected against *Lagenidium myophilum*, a known shrimp pathogen, by entering into an association with a symbiotic bacterium.

To determine if the bacterial flora protects the eggs from fungi, clusters of eggs were removed from four *C. septem-*

spinosa females, and equally divided into four groups of about 50 eggs. Each of these egg groups was placed into a separate, sterile petri dish containing 25 ml sterile filtered seawater (SFSW), and observed daily under a dissecting microscope. The control group received no treatment and was identified as A(polymyxin B(-)/*L. myophilum*(-)). Any surface-dwelling bacteria were eliminated from two of the three remaining groups by treatment of the bathing SFSW with polymyxin B sulfate (0.1 mg/ml). To monitor the sole effect of the antibiotic on the eggs, one group received no further treatment after the exposure to polymyxin B B(polymyxin B(+)/*L. myophilum*(-)). The remaining two groups (1 polymyxin B-treated, 1 non-treated) were introduced a 1-ml inoculum of a thick liquid culture of the pathogenic fungus *L. myophilum*; C(polymyxin B(-)/*L. myophilum*(+)), D(polymyxin B(+)/*L. myophilum*(+)). Each day, each group of eggs was classified as having no infection, some infection, or a heavy infection. At the end of the four-day trial, only the group of eggs that was (polymyxin B(+)/*L. myophilum*(+)) was significantly colonized by *L. myophilum* (Fig. 1).

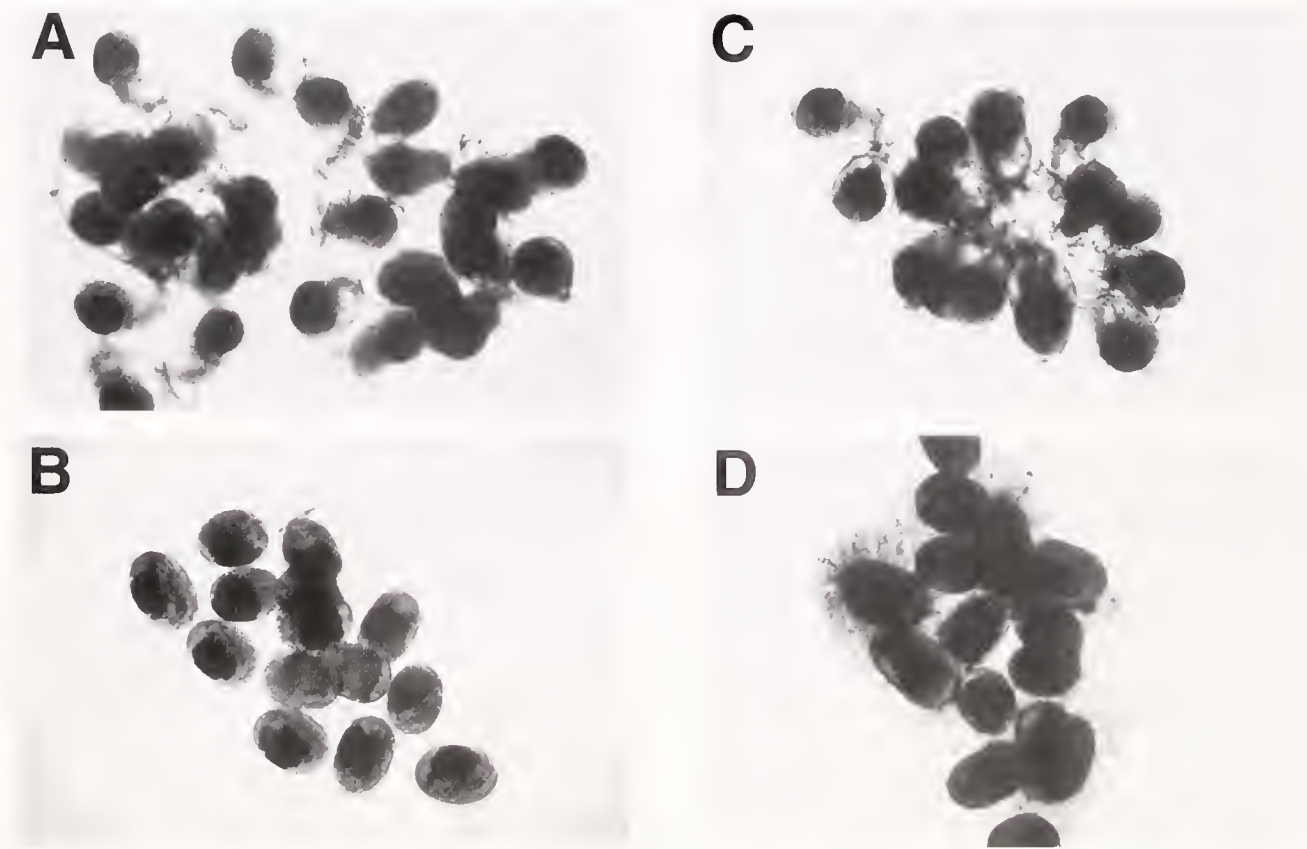


Figure 1. Surface-dwelling bacteria on the eggs of *Crangon septemspinosa* provide a defense against *L. myophilum*. (A) Representative native egg cluster (B) Native egg cluster post four-day inoculation of the fungus *L. myophilum*. (C) Native egg cluster after treatment with Polymyxin B. (D) Treated egg cluster post four-day inoculation of *L. myophilum*.

Antifungal testing of bacteria isolated from the egg surface was performed by standard inhibition diffusion assays on a dilute concentration of Difco Marine agar 2216 (4). One distinct bacterium, a copper-colored, fast-growing, gram-negative strain, consistently showed >2 mm inhibition from the bacterial plug after 1 day, whereas all other bacterium isolates showed ≤ 1 mm inhibition. In liquid culture, this bacterium also inhibited the growth of *L. myophilum*. Aside from potential competition for nutrients and adhesion sites at the egg surface, this study demonstrates inhibitory action by an epibiotic bacterium against a related organism through the release of specific antagonistic chemical compounds. We intend to further

analyze the chemical nature of the compounds secreted by surface-dwelling bacteria inhabiting the surface of externally brooded eggs of marine invertebrates and study potential ecological implications.

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Abundance and Age-Specific Growth Rates in Relation to Population Densities of *Fundulus heteroclitus* in Waquoit Bay Estuaries Subject to Different Nitrogen Loads

Erin LaBrecque, Cara Fritz, Joanna Tober, Peter J. Behr, and Ivan Valiela (Boston University Marine Program, Marine Biological Laboratory)

Coastal environments throughout the world are increasingly enriched with nitrogen (1), but there is only rudimentary knowledge about how nitrogen loading affects fish communities. The estuaries of Waquoit Bay, Massachusetts, receive different nitrogen-loading rates so that they provide the opportunity to examine how fish communities respond to a range of nitrogen-loading rates. Our objectives were to define how population density and age-specific growth rates of the salt-

marsh killifish, *Fundulus heteroclitus*, were related to different nitrogen-loading rates.

F. heteroclitus was chosen for this study for three reasons. First, it is the most abundant fish in Waquoit Bay (2). Second, food availability, and the related issue of population density, limit its growth rates (3). Third, it has a relatively small annual home range (4, 5) so that populations are likely to remain within a single estuary during their life spans.

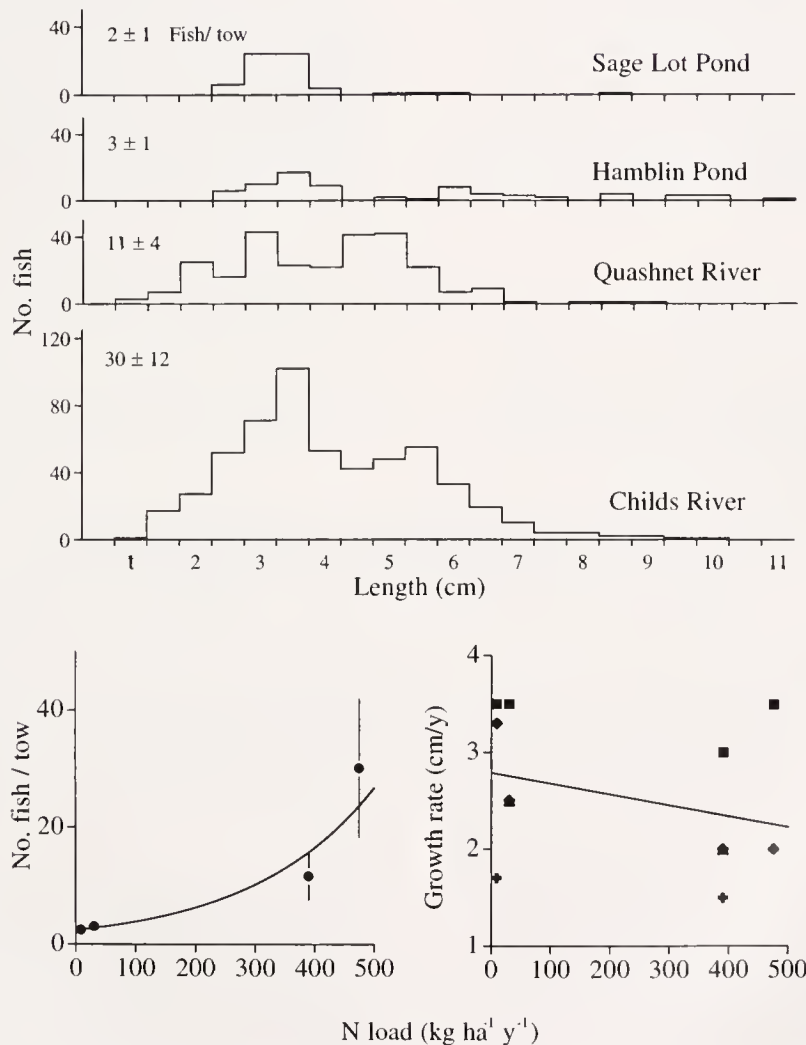


Figure 1. (Top four graphs) Length-frequency histograms of *Fundulus heteroclitus* in Sage Lot Pond, Hamblin Pond, Quashnet River, and Childs River. Numbers on top left of each histogram indicate number of fish per two ± SE. (Bottom left) Abundance of *F. heteroclitus* (±SE) in relation to different nitrogen-loading rates ($y = 2.41 \cdot 10^{0.002x}$, $r^2 = 0.96$). (Bottom right) Average growth rates for age groups 1–4 of *F. heteroclitus* in relation to the nitrogen-loading rates (regression $F = 1.52$, $P = 0.24$). Year 1: squares; year 2: diamonds; year 3: crosses; year 4: triangles.

Fish were sampled in four estuaries of Waquoit Bay with different nitrogen loading: Sage Lot Pond ($9 \text{ kg N ha}^{-1} \text{ y}^{-1}$), Hamblin Pond ($30 \text{ kg N ha}^{-1} \text{ y}^{-1}$), Quashnet River ($390 \text{ kg N ha}^{-1} \text{ y}^{-1}$), and Childs River ($475 \text{ kg N ha}^{-1} \text{ y}^{-1}$). We performed seine tows 10 m along the shore with a 5-m-wide seine net at five sites within each estuary, and measured standard lengths of the *F. heteroclitus* caught. To age the fish, we collected scales on the lateral line, behind the operculum (6).

Abundance of *F. heteroclitus* increased as nitrogen loading increased (regression, $F = 10.71$, $P < 0.01$) (Fig. 1, numbers on top left of top four panels, and Fig. 1 bottom left). Although the population density of *F. heteroclitus* increased with nitrogen loading, the median size of the fish did not vary (regression, $F = 0.61$, $P = 0.44$) [data not shown].

We calculated growth rates of each of the four year classes of *F. heteroclitus* using the modal length of each age class found from length-frequency histograms (Fig. 1, top four graphs) and standard-deviate determination of age cohorts (7). For example, the growth rate for age 2 was determined by subtracting the modal length of age 1 from the modal length of age 2. Growth rates of the four age classes of *F. heteroclitus* were variable, but appeared to be somewhat lower—or at least did not increase—as nitrogen-loading rates increased (Fig. 1, bottom right).

The data suggest that there are more *F. heteroclitus* in the more nitrogen-loaded estuaries, even though surveys in Waquoit Bay show that abundance of potential prey in estuaries subject to high nitrogen loads is half that of unenriched estuar-

ies (J. McClelland, Boston University Marine Program, pers. comm.). For *F. heteroclitus*, however, the lower prey densities must still be sufficient to sustain—or only slightly reduce—growth rates. Thus the populations of *F. heteroclitus* in Waquoit Bay do not seem to be food limited. We lack information about what determines abundance of this species in Waquoit Bay, but one possible explanation for its increased abundance in estuaries subject to higher nitrogen-loading rates may be that higher nitrogen loads somehow lead to increased recruitment or immigration. This possibility needs further examination.

This work was supported by funds from a grant from the NSF Research Experience for Undergraduates Program (E.L.) and the Woods Hole Marine Science Consortium (C.F., J.T.).

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Assessment of Fish Communities in New England Embayments: Application of the Estuarine Biotic Integrity Index

Kehaulani Chun (Brigham Young University), Melissa J. Weaver¹, and Linda A. Deegan¹

The Estuarine Biotic Integrity (EBI) index was developed to evaluate the status of estuarine ecosystems on the basis of their fish communities (1). Fish depend on estuarine habitats for food, refuge from predation, spawning, and rearing of young. Nitrogen loading associated with upland development disrupts these functions and results in an impoverished fish community (1). The EBI index is based on metrics derived from the fish community and complements chemical, physical, and other biotic indicators in determining the status of an ecosystem.

We used the EBI index to evaluate interannual and long-term (5 to 7 years) variability of ecosystem status in two New England estuaries (Waquoit and Buttermilk Bays). We expect to see smaller differences between 1988 and 1989 and between 1995 and 1996 (reflecting natural variability) compared to

differences between the two sets of years (reflecting long-term changes in anthropogenic stress). We anticipate seeing less change in Buttermilk Bay than in Waquoit Bay due to a reduction in the amount of nitrogen input to Buttermilk Bay following new regulations of land use and control of storm drain runoff (2).

Fish were collected from six trawl hauls per site during July and August, and the EBI value was calculated for each site (1). Sites were classified as having low or medium habitat quality on the basis of 1988 abiotic variables (1). The EBI index is a function of eight variables: number of species; dominance; number of resident species; number of nursery species; number of estuarine spawners; abundance; proportion of benthic fishes; and proportion with physical abnormalities (1). Abundance and proportions were based on either number of individuals or biomass. The EBI values based on number and on biomass were very similar and highly correlated ($r^2 = 0.90$, $F = 2171.2$,

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$P < 0.0001$) suggesting that they are closely linked and respond similarly to environmental conditions (Fig. 1). The EBI index is normally distributed (1); therefore, significant differences ($P < 0.05$) were based on contrast analyses following a three-way, cross-nested ANOVA using estuary, year, and habitat quality as main effects. Sites were nested within habitat quality, and the number of sites within a quality group determined the degrees of freedom for the F -value.

Interannual differences were found in Waquoit Bay but not in Buttermilk Bay. In Buttermilk Bay, the EBI index was similar between 1988 and 1989 and between 1995 and 1996 for both low- and medium-quality habitats. In Waquoit Bay, EBI values were similar between 1995 and 1996 for both low- and medium-quality habitats, but the value for low-quality habitats declined between 1988 and 1989 ($F = 21.4$, $P = 0.04$). This difference may be a result of a fish kill that was noticed in 1989. The EBI values for 1988 and 1989 were both below the critical values for designating a habitat as low in quality (1). EBI values for low-quality habitats were not different between 1995 and 1996, and these were most similar to those in 1989.

Over the long term, the EBI index was stable in Buttermilk Bay but declined in the medium-quality habitats in Waquoit Bay. Efforts to control nitrogen inputs into Buttermilk Bay may have prevented further degradation (2). In Waquoit Bay, the EBI value declined in medium-quality habitats (EBI biomass, $F = 101.5$, $P < 0.01$, EBI number, $F = 14.3$, $P = 0.06$) to values equivalent to those of low-quality habitats. Medium-quality habitats may have been degraded to low quality by continued anthropogenic stress.

The EBI index indicates changes in habitat quality due to efforts made to improve estuaries or to continuing stress. It reflects the integrated effects of several abiotic and biotic variables that may either singly or in combination affect the ability of estuarine habitats to support fish. The next step would be to search for changes in these abiotic and biotic variables to pinpoint the cause of the observed change in EBI values.

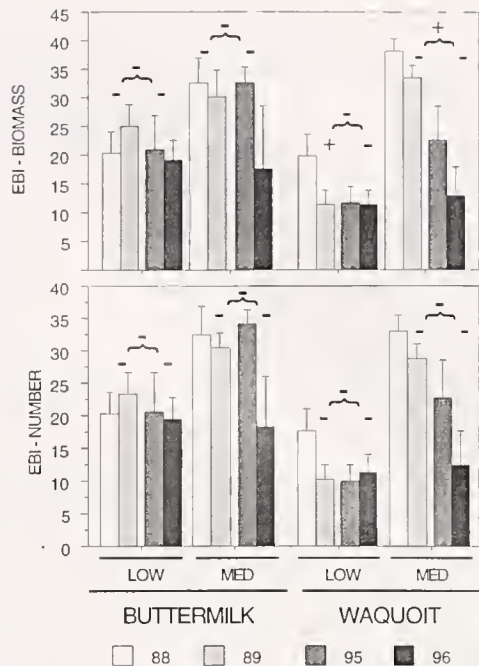


Figure 1. Interannual and long-term variability of the Estuarine Biotic Integrity (EBI) index in Waquoit and Buttermilk Bays. (—) represents no difference and (+) represents a significant difference.

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Abundance, Biomass, and Species Richness of Fish Communities in Relation to Nitrogen-Loading Rates of Waquoit Bay Estuaries

Joanna Tober, Cara Fritz, Erin LaBrecque, Peter J. Behr, and Ivan Valiela (Boston University Marine Program, Marine Biological Laboratory)

Nitrogen loading may increase primary production (1), and as primary production increases, fish yield may also increase (2). To associate fish stocks with nitrogen inputs in specific estuaries, we examined the relationship between nitrogen loading and abundance, species richness, biomass, and length distribution of fish in Waquoit Bay, Massachusetts. We chose four Waquoit Bay estuaries (Sage Lot Pond, Hamblin Pond, Quashnet River, and Childs River) that are subject to different nitrogen loads (9, 30, 390, and 475 kg N ha⁻¹ y⁻¹, respectively).

We collected fish with a 5-m seine net by towing for 10 m along the shore at five sites in each estuary, and at five dates

during June and July of 1996. After each tow, we identified, counted, and measured the fish caught, keeping 50 of each species for dry weight measurements.

Fish abundance increased as nitrogen load increased (regression $F = 15.97$; $P < 0.01$) (Fig. 1, top). The average number of species per tow also increased as nitrogen load increased (regression $F = 29.35$; $P < 0.01$) (Fig. 1, top). Our results directly support previous findings that link increased fish yield with increased nitrogen load (2). The increase in species richness is not, however, as predicted by other studies (3).

We converted fish counts into biomass by using length-

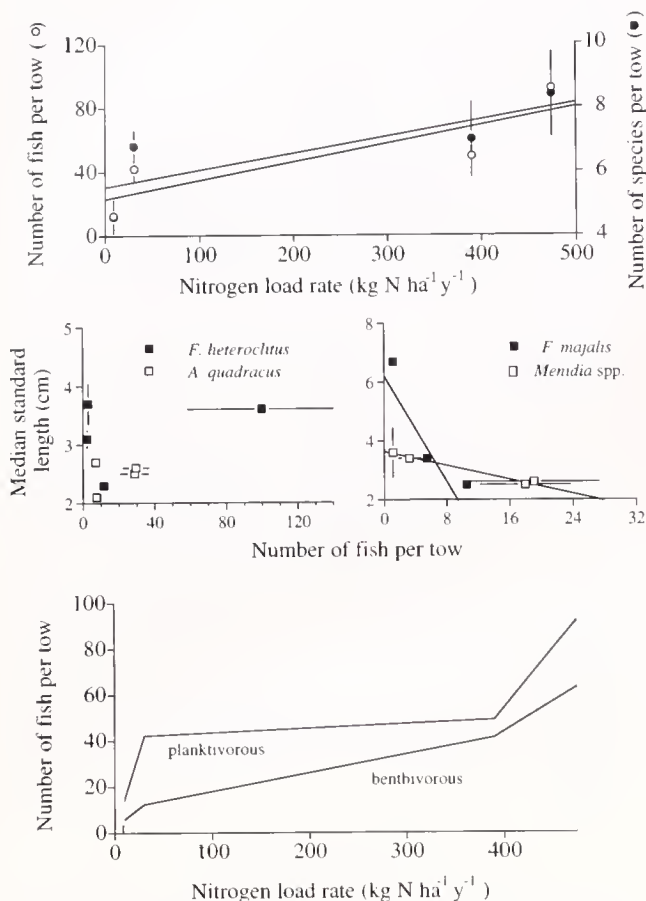


Figure 1. Total number of fish per tow $\pm$ SE (top panel, left axis) and total number of species per tow $\pm$ SE (top panel, right axis) are plotted versus nitrogen loading (number of fish: $y = 0.114x + 23.093$, $r^2 = 0.712$; number of species: $y = 0.005x + 5.521$, $r^2 = 0.641$). Median length of *Fundulus heteroclitus* and *Apeltes quadracus* $\pm$ SE is plotted versus the total number of fish per tow $\pm$ SE (middle left panel). Median length of *Menidia* spp. and *Fundulus majalis* $\pm$ SE is plotted versus the total number of fish per tow (middle right panel). Regression lines shown for graphs of median length (middle panels) are geometric mean regressions (8) (*Menidia* spp.: $y = -0.058x + 3.627$, $r^2 = 0.982$, and *F. majalis*: $y = -0.447x + 6.202$, $r^2 = 0.637$). The number of planktivorous and benthivorous fish are stack-plotted versus nitrogen loading (bottom panel).

weight regressions for the species collected in our tows. Total biomass of all the species was not related to nitrogen load (regression $F = 1.37$; $P = 0.24$) (data not shown). These results suggest that the fish of Waquoit estuaries are more numerous

as nitrogen load increases, but that the fish in the more enriched estuaries must be smaller. Higher fish densities could result in smaller fish if food supply were limited.

To determine whether lengths were reduced where fish were denser, we plotted median length of the four major taxa, *Fundulus heteroclitus*, *Fundulus majalis*, *Menidia* spp., and *Apeltes quadracus*, versus total density of fish (Fig. 1, middle two panels). In two of the four major species (Fig. 1, middle left) length was not related to density, but in the others it was (Fig. 1, middle right). *F. majalis* feeds on bare sand-silt areas (4), which are much reduced in estuaries subject to high nitrogen loads where macroalgal cover increases (5). *Menidia* spp. also showed lower size as density increased; this species differs from the other taxa in that it is a planktivore (4).

We classified the different fish species as either benthivorous or planktivorous (4, 6) and examined how the abundance of these two trophic categories may be related to nitrogen load (Fig. 1, bottom). The planktivorous fish (mainly *Menidia* spp.) did not change in abundance, but the benthivorous fish, primarily *F. heteroclitus*, increased steadily with nitrogen load. The relationship to nitrogen-loading rate is apparent, but in all comparative studies such as this one, other features associated with the different estuaries might be important; here we chose to emphasize the likely link to nitrogen-loading rate.

Studies done in the Bay have shown that the density of benthic invertebrates is two times greater in the lowest nitrogen-loading estuary than in the highest nitrogen-loading estuary (McClelland, J., Boston University Marine Program, pers. comm.). In earlier papers, decreases in fauna have been tentatively attributed to a higher frequency of hypoxic and anoxic events (7). The data presented here alternatively suggest that the larger abundance of benthivorous fish might be partially responsible for the decrease in benthos as nitrogen load increases.

This work was supported by the funds for a grant for NSF Research Experience for Undergraduates Program (E.L.), and by the Woods Hole Marine Sciences Consortium (C.F., J.T.).

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Selection of Nitrogen-Enriched Macroalgae (*Cladophora vagabunda* and *Gracilaria tikvahiae*) by the Herbivorous Amphipod *Microdeutopus gryllotalpa*

Eris Galán Jiménez, Jennifer Hauxwell, Elizabeth Heckscher, Carol Rietsma, and Ivan Valiela
(Boston University Marine Program, Marine Biological Laboratory)

Marine consumers are limited by nitrogen (1), so herbivores would benefit from the ability to detect differences in % nitrogen in their food choices. Because nitrogen enrichment of producers in coastal ecosystems is increasing world-wide (2), herbivores in many coastal systems may find themselves with N-enriched foods. To determine whether herbivores respond to increased nitrogen content, we ran experiments in which the herbivorous amphipod *Microdeutopus gryllotalpa* was given choices of same species-macroalgal foods from two estuaries of Waquoit Bay, Cape Cod, MA, which are subject to different nitrogen-loading rates; Childs River receives a relatively high nitrogen-load ($470 \text{ kg N ha}^{-1} \text{ y}^{-1}$), while Sage Lot Pond is relatively pristine ($10 \text{ kg N ha}^{-1} \text{ y}^{-1}$) (3).

To establish that macroalgae from the two estuaries show differences in nitrogen content, we first determined the % nitrogen in *Cladophora vagabunda* and *Gracilaria tikvahiae* from both estuaries. These species comprise over 85% of the total macroalgal biomass in both estuaries (4) and probably serve as important food sources for herbivores in Waquoit Bay. Algae were collected in late July 1996, sorted, cleaned of debris, rinsed with fresh water, dried for 24 h at 60°C , and ground with mortar and pestle. Between 2 and 4 replicate samples were run on a model 2400 Perkin Elmer CHN analyzer. Algae from the high nitrogen-loaded estuary contained significantly ($1.3\text{--}1.5\times$) more N than the same algae from the more pristine estuary (Table 1, top).

The amphipod *Microdeutopus gryllotalpa* is the most abundant herbivorous crustacean in Waquoit Bay [up to >2000 individuals m^{-2} , (4)] and is a tube-dweller that probably consumes the algae in which it resides. We used *M. gryllotalpa* in choice experiments in which number of amphipods found within same-species algal fronds from either Childs River or Sage Lot Pond served as a proxy for overall preference, which includes preference for residence as well as food (microhabitat-choice experiments). In addition, to test whether grazers are habituated to feed on algae from their own estuary, we used amphipods from each estuary in separate experiments. ✓

We placed either *C. vagabunda* or *G. tikvahiae* (amount equivalent to 1 ml volume displacement, anchored with small glass weight) from both estuaries in $21 \times 21 \times 12 \text{ cm}$ plastic containers containing 3.7 l of aerated 20°C seawater (35‰). Four petri dishes (5 cm diam.) were placed in each container; two dishes contained algae from Childs River and two contained algae from Sage Lot Pond. We placed 30 adult (4–6 mm) *M. gryllotalpa* in the middle of each container and allowed them 2 days to choose and move into preferred algae. Throughout the experiment, we maintained 12 h light/12 h dark cycles to simulate natural conditions. After 2 d, we placed lids over each petri dish, removed the dishes from the container, and recorded amphipod density in each dish. Five to six replicates were run. Regardless of estuary of origin, amphipods preferen-

Table 1

Top: Nitrogen content of the macroalgae *Cladophora vagabunda* and *Gracilaria tikvahiae* from enriched (Childs River) and more pristine (Sage Lot Pond) estuaries of Waquoit Bay. Middle: Results of pairwise choice experiments using either *C. vagabunda* or *G. tikvahiae* from Childs River and Sage Lot Pond to assess microhabitat preference of *Microdeutopus gryllotalpa* (mean number of amphipods in algae from the same estuary and % of total $\pm$ SE). Bottom: Feeding preference of *M. gryllotalpa* for algae from Childs River or Sage Lot Pond (mean number of bite marks on algae from the same estuary and % of total $\pm$ SE). Asterisks indicate significance of paired t-tests at $P = 0.05$ for number of amphipods within algae and percentages of bite marks on algae

Nitrogen Content					P-value
% nitrogen:	Childs River		Sage Lot Pond		
<i>C. vagabunda</i>	4.52 ± 0.07		3.40 ± 0.07		0.001*
<i>G. tikvahiae</i>	2.09 ± 0.04		1.38 ± 0.09		0.003*
Habitat Choice					
Amphipods found within algae:					
	Number	% of total	Number	% of total	
Childs River amphipods					
<i>C. vagabunda</i>	21.2 ± 1.8	82 ± 4	4.7 ± 1.1	18 ± 4	0.001*
<i>G. tikvahiae</i>	15.2 ± 2.1	62 ± 6	9.2 ± 1.4	38 ± 6	0.147
Sage Lot Pond amphipods					
<i>C. vagabunda</i>	17.2 ± 3.5	73 ± 9	5.2 ± 1.5	27 ± 9	0.050*
<i>G. tikvahiae</i>	18.0 ± 1.3	68 ± 5	8.6 ± 1.4	32 ± 5	0.025*
Food Choice					
Bite marks:					
<i>C. vagabunda</i>	534 ± 180	82 ± 5	142 ± 71	18 ± 5	0.005*
<i>G. tikvahiae</i>	1539 ± 270	75 ± 6	463 ± 116	25 ± 6	0.031*

tially inhabited nitrogen-enriched *C. vagabunda* and *G. tikvahiae* (Table 1, middle).

To determine whether results from the microhabitat-choice experiments were due to preferential consumption of nitrogen-enriched algae, we conducted additional experiments in which we measured feeding by amphipods on agar suspensions (5) of the same species of macroalgae from different estuaries. These feeding-choice experiments also eliminate the effect of morphological or toughness differences between same species of algae from different estuaries. To prepare agar suspensions (5), we used 2.4 g (wet weight) of chopped *C. vagabunda* or *G. tikvahiae* from each estuary, poured into two opposing quarters

of four-compartment petri dishes (8.5 cm diameter). Eight amphipods (from Sage Lot Pond) were placed in each petri dish and allowed 12 h to graze. We then counted the number of bite marks on the agar surface in each compartment as an indication of food preference. Four replicates were run for each species of algae. Amphipods preferentially fed upon nitrogen-enriched algal suspensions (Table I, bottom).

Food selection by herbivorous amphipods is clearly correlated with nitrogen content (Table I); *M. gryllotalpa* exhibited significant preference even when differences in nitrogen content were as small as 0.7% (Table I, top, difference in % N of *G. tikvahiae*). Preference may not be solely due to nitrogen content; other chemical cues (secondary compounds) may be correlated to nitrogen content of macroalgae and may also be important to herbivores.

Differences in chemical deterrents may be present in same algal taxa from estuaries of different nitrogen-loading rates where the same deterrent compound exists, but in different concentrations. For instance, in a study of snail grazing on *Fucus vesiculosus* collected from the same two estuaries of Waquoit Bay, Yates and Peckol (6) found that snails preferred N-enriched algae from Childs River over Sage Lot Pond. They also found that the concentration of defensive polyphenolic compounds was significantly lower in N-enriched algae. Differences in chemical deterrents may also exist among different algal taxa where different compounds are present. Heckscher *et al.* (7) found in similar agar feeding experiments comparing *M. gryllotalpa* feeding preference between *C. vagabunda* (4.5% N) and *G. tikvahiae* (2.1% N) from Childs River,

that *G. tikvahiae* was preferred; in that case, a chemical cue besides nitrogen content affected palatability. Therefore, it is likely that, in the absence of morphological cues, nitrogen as well as defensive compounds determine feeding preferences of herbivores (5).

Nitrogen-loading to estuaries results in changes in the taxonomic composition of producer communities and overall increased macroalgal biomass (2), as well as increased tissue N content of macroalgae (Table I, top). By affecting what type of algal food is present, the amount of algae that is present, and their N content, estuarine enrichment should therefore alter the interactions between herbivores and their foods.

This work was supported by an NSF Research Experience for Undergraduates Program internship to E.G.J. and a Woods Hole Marine Consortium internship to E.H. Special thanks to K. Foreman for assistance with the CHN analyses.

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Selectivity by the Herbivorous Amphipod *Microdeutopus gryllotalpa* Among Five Species of Macroalgae

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The amphipod *Microdeutopus gryllotalpa* is the most abundant mesograzer in estuaries of Waquoit Bay, Massachusetts, reaching densities of 2000 individuals m^{-2} (1). Though models suggest that amphipod grazing pressure can greatly influence macroalgal biomass (1), there is little information about whether *M. gryllotalpa* eats all or some of the species of macroalgae found in Waquoit Bay. Different grazing pressures could shift the dominance of these macroalgal species.

Amphipods may select food items based on chemical cues (nutrition, feeding deterrent compounds) and morphology (provision of cover) (2). We compared the selectivity of *M. gryllotalpa* towards different macroalgal taxa (*Enteromorpha* spp., *Ulva lactuca*, *Cladophora vagabunda*, *Gracilaria tikvahiae*, and *Polysiphonia* spp.) collected from the Childs River estuary of Waquoit Bay and examined the relative importance of chemical and morphological cues.

To test selectivity among different algal taxa, we presented amphipods with a choice between two species of algae. We placed four petri dishes containing a pair of one taxa and a pair of another taxa (each held in place by a clear glass weight) into a 4-liter plastic container filled with aerated seawater (20°C, 35‰), and then released 30 amphipods. All experiments were run for 12 h light/12 dark cycles. After 48 h we placed lids on each petri dish, removed them from containers, and counted the amphipods in each species of algae. We ran five replicates of each possible algal comparison and measured preference as the difference between the number of amphipods on different species.

The pair-wise tests showed that *M. gryllotalpa* discriminated among the algal taxa (Fig. 1, top), preferring *Polysiphonia* spp. in all tests; *U. lactuca* and *G. tikvahiae* were least preferred.

		Filamentous			Branched
		<i>Enteromorpha</i> spp. (1.1 % N)	<i>C. vagabunda</i>	<i>Polysiphonia</i> spp.	<i>G. tikvahiae</i>
Filamentous	<i>C. vagabunda</i> (4.4 % N)	14.8 ± 1.4	--	--	--
	<i>Polysiphonia</i> spp. (2.3 % N)	12.6 ± 1.2	9.2 ± 0.5	--	--
	<i>G. tikvahiae</i> (2.1 % N)	15.9 ± 1.9	16.8 ± 0.6	19.8 ± 1.3	--
Branched	<i>U. lactuca</i> (1.7 % N)	6.0 ± 1.4	9.0 ± 1.0	6.8 ± 1.2	--
Bladed	<i>U. lactuca</i> (1.7 % N)	16.0 ± 1.8	16.8 ± 1.4	15.6 ± 3.0	13.4 ± 1.9
	<i>U. lactuca</i> (1.7 % N)	8.4 ± 1.2	10.0 ± 1.1	9.6 ± 2.2	10.6 ± 1.6

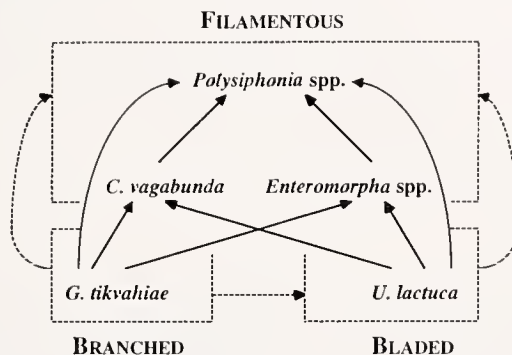


Figure 1. Results of overall preference and morphology experiments. Top: pair-wise comparisons of algal species; numbers beneath each algal species indicate its percent N composition; numbers in the table are the number of amphipods residing in each different macroalga (mean $\pm$ SE). Bottom: hierarchy summary in which arrows point towards the preferred macroalgal species and preferred mimic morphology; solid lines represent the preference hierarchy and dashed lines represent the morphology hierarchy; significance determined by replicated goodness of fit G-tests (7).

Although filamentous algae were selected over branched and bladed algae, it is unclear in these experiments whether the choice was due to morphology or chemistry.

To examine whether these preferences were due to chemical cues, we ran palatability tests (3). To eliminate morphological differences among algae, 2.4 g of chopped algae were suspended in 100 ml of a 1.6% agar solution. Divided petri dishes containing suspensions of two species of algae were placed in flow-through containers and submerged in running seawater. Eight amphipods were allowed to feed in the dark for 12 h. Eight replicates for each comparison were run, and feeding preference was assessed by counting bite marks on each of the two species in each dish.

The macroalgae tested were equally palatable to *M. grylotalpa*. Out of 10 pair-wise comparisons, we found only one significant difference from an expected 1:1 ratio (paired *t*-test). In similar tests using same-species comparisons between the same algal species, Galán Jiménez *et al.* (4) were able to correlate palatability with % N, and amphipods detected differences in N content as small as 0.7%. Although the algae tested ranged from 1.1% to 4.4% N (Fig. 1, top), we found no correlation between N and palatability. The algae with the highest % N also potentially have secondary compounds (*e.g.*, halogenated aromatics in *Cladophora* spp., acetogenins in *Gracilaria* spp., and halophenolics in *Polysiphonia* spp.) (5) that may account for lack of correlation with N content. In our experiment, *G. tikvahiae*—whose % N is lower (2.1% N) (Fig. 1, top) than that of *C. vagabunda* (4.4% N) (Fig. 1, top)—was preferred (mean % of bites

92 $\pm$ 7), indicating that % N alone did not account for differences in palatability. Overall, these results indicate that chemical cues are not the basis for the selectivity *M. grylotalpa* exhibited in the initial preference experiment.

To eliminate chemical differences and examine importance of frond morphology, we ran algal mimic experiments. Mimics simulated filamentous morphology with interstitial spaces ranging between 0 and 1 mm (balled-up plastic mesh), branched morphology with spaces from 5 to 10 mm (plastic aquarium plants), and bladed morphology (green plastic wrap). Two pairs of mimic algae were placed in containers; methodological details are similar to those of the first experiment.

In same-species comparisons of Galán Jiménez *et al.*, where morphology is not a variable, N content was correlated with preference (4). When, however, amphipods were presented with different taxa of algae, they chose primarily on the basis of morphology; filamentous mimics were preferred significantly (Fig. 1) over bladed (16 $\pm$ 2:9 $\pm$ 2) and branched mimics (18 $\pm$ 4:4 $\pm$ 2). *M. grylotalpa* was also observed to build more tubes in live filamentous algae. Morphology is therefore an important factor explaining our preference hierarchy (Fig. 1, bottom).

Our experiments show that the major taxa of algae were equally palatable to *M. grylotalpa*. These results imply that *M. grylotalpa* might graze equally on the five algae tested. The amphipods' demonstrated preference for filamentous morphology (Fig. 1, bottom) suggests, however, that algae of this form attract more amphipods and, at least in the case of the

tube-dweller *M. gryllotalpa* where the host algae is also food, may be subject to higher grazing pressure. Carbon and nitrogen stable isotope studies of the food web in Waquoit Bay corroborate these results and indicate that, of the two major species (>85% of total biomass) of algae in the Bay (*G. tikvahiae* and *C. vagabunda*), the filamentous alga *C. vagabunda* comprises a large portion of the diet of *M. gryllotalpa* (6).

This work was supported by a Woods Hole Marine Science Consortium internship to E. H. and an NSF Research Experience for Undergraduates internship to E. G. J. Special thanks to K. Foreman for assistance with CHN analysis.

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Shrimp in Waquoit Bay: Effects of Nitrogen Loading on Size and Abundance

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Shrimp play a significant role in estuaries as epibenthic omnivores, consuming plant detritus and providing protein-rich waste and potential food items for other trophic levels (1). In estuaries like Waquoit Bay, Massachusetts, an increase in nitrogen loads results in a greater abundance of macroalgae (2), which in turn may influence shrimp populations. Our study examined the effects of nitrogen loading on the abundance and size of shrimp in Waquoit Bay.

Two species of grass shrimp, *Palaemonetes pugio* and *P. vulgaris*, and the sand shrimp *Crangon septemspinosus* were collected from four estuaries of Waquoit Bay. The estuaries, Sage Lot Pond, Hamblin Pond, Quashnet River, and Childs River, represent a gradient of nitrogen loading, with modeled annual nitrogen-loading rates of 9, 30, 390, and 474 kg N ha⁻¹, respectively. Using a 5-m-long seine net, we towed for a distance of 10 m along the shore. Collections were made five times during June and July 1996, at five locations in each estuary. Carapace lengths were measured on all specimens of *P. pugio* and *P. vulgaris* collected. Length of *C. septemspinosus* was taken as the distance from eye socket to base of telson. A total of 6086 shrimp were collected from the four estuaries.

The average number of shrimp per tow increased significantly with increased nitrogen loading when all points are plotted (regression $F = 7.12$, $P < 0.01$) (Fig. 1, top panel). Response to nitrogen loading varied with species (Fig. 1, bottom left panel). *P. pugio* was the most abundant shrimp over the nitrogen-loading gradient; *P. vulgaris* became common at the highest nitrogen loads. Because *P. pugio* showed a steady increase in number as nitrogen loading increased, it may be a useful indicator of eutrophication.

Using length frequency histograms and standard deviates (3)

we detected three age groups in each of the three species. We show only the length of each age class for *C. septemspinosus* in Fig. 1 (bottom right panel) since the data for *P. pugio* and *P. vulgaris* followed similar trends. Age groups 2 and 3 show a slight downward slope in size as nitrogen-loading rate increases. Nonetheless, because each point is the mean of many individual measurements (5 to 370 individuals per mean) and the regression was calculated using values for all shrimp, the slopes of lines shown are statistically significant (regression F range 76.39–93.39, $P < 0.01$).

There is an indication that increased nitrogen load decreased the size of young shrimp, but this effect was lost as cohorts matured. Age group 1 for each species tended to be smaller in size as loading increases. As shrimp age, this decrease in size in response to nitrogen loading disappears; by the time shrimp enter age group 3, there was little detectable effect of nitrogen loading. One possible explanation for this smaller size may be that the younger shrimp are sensitive to the more frequent hypoxic events in the higher nitrogen-loaded estuaries (4).

This work was supported by funds from a grant from the NSF Research Experience for Undergraduates (E. L.) program and by the Woods Hole Marine Science Consortium (C. F., J. T.).

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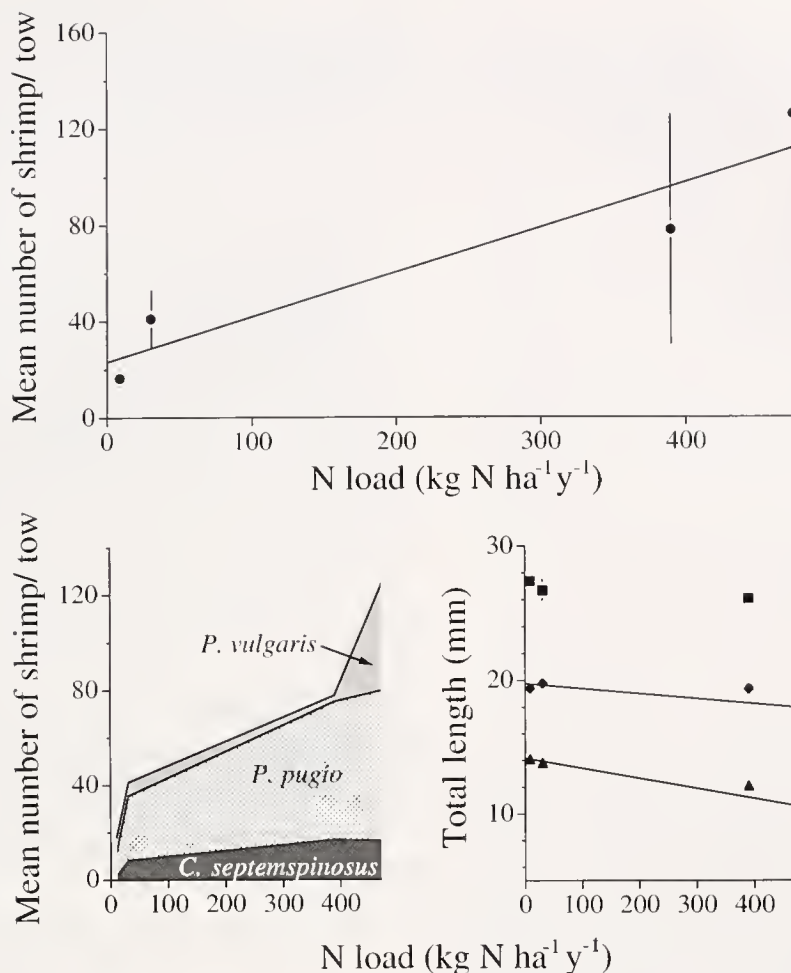


Figure 1. Average number of shrimp collected per seine tow $\pm$ SE is plotted as a function of nitrogen loading (top panel). Species composition is stack-plotted as a function of increasing nitrogen loading (bottom left panel). Average total length $\pm$ SE for each of three age classes of *C. septemspinosus* is plotted versus nitrogen loading (bottom right panel). Straight lines are included only when the regression of length versus nitrogen load was significant. Age group 1: triangles; age group 2: diamonds; age group 3: squares.

Reference: *Biol. Bull.* **191**: 327–328, (October, 1996)

Growth Rates of Ribbed Mussels in Six Estuaries Subject to Different Nutrient Loads

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Atlantic ribbed mussels (*Geukensia demissa*) inhabit the intertidal zone in estuarine salt marshes and feed by filtering phytoplankton and particulate organic matter (POM) from the water (1). Nutrients from anthropogenic sources are stimulating production of organic matter in estuaries worldwide (2). To test the possible effect of nitrogen loading on the growth of ribbed mussels, we examined age-specific growth rates of mussel populations growing in six estuaries of Waquoit Bay, Massachusetts, that are subject to different nitrogen loads. We hypothesized that growth of mussels depends on food supply, therefore

increased organic matter production due to nitrogen loading should accelerate mussel growth.

We sampled 35–50 mussels from each estuary, each sample representing the range of shell length (>8 mm) and age (>1 year) of the source estuary. Mussels were taken from creek bank in the 15-cm band directly below the roots of *Spartina alterniflora*. *S. alterniflora* grows at a consistent tidal height, so sampling in this band allowed us to sample at an equal tidal height in all estuaries. We measured the length of each mussel and determined its age by counting internal growth rings (3).

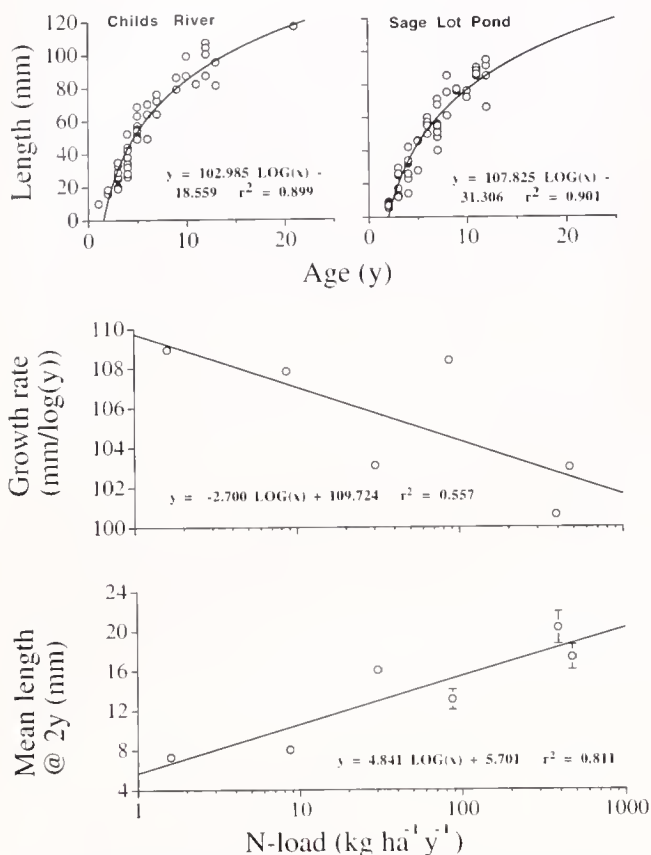


Figure 1. Age versus length curves for Waquoit Bay estuaries with high (top left) and low (top right) N loads (estuaries sampled were Childs River, Quashnet River, Eel Pond, Hamblin Pond, Sage Lot Pond, Timm's pond), growth rate versus N load ($P = 0.0882$) (middle) and mean length at 2 years of age versus N load (bottom). N-loading figures are modeled values taken from Waquoit Bay Land Margins Ecosystem Research unpublished data. Error bars on the bottom panel represent 1 standard error; missing bars are too small to appear on the graph.

We approximated a growth rate for each estuary by fitting a logarithmic curve to a plot of length versus age (Fig. 1, top). Logarithmic growth, fastest at young ages and declining steadily thereafter, is prevalent among bivalves (4). We used the log coefficients of these curves, which represent rates of growth in millimeters/log (year), to compare growth between estuaries. To obtain an independent check on the calculated growth rates, we also experimentally determined growth rates over a short period of time by planting mussels (15–35 mm, 2–4 years of

age) in two estuaries, one with high nitrogen loading (high N) and the other with low loading (low N), and measuring change in shell length after 2 weeks.

Contrary to our hypothesis, growth rates for the entire mussel population did not increase with N loading; instead, they decreased ($P = 0.088$, $r^2 = 0.56$) in estuaries with greater N loads (Fig. 1, middle). This unexpected finding was corroborated by the experimental results: mussels planted in the low-N estuary grew significantly faster (0.04 mm/day) ($P = 0.026$) than mussels planted in the high-N estuary (0.01 mm/day).

The data on which we based our calculated growth rates did not, however, include mussels younger than 2 years of age. The size of year 2 mussels increases with N loading (Fig. 1, bottom) ($P = 0.014$, $r^2 = 0.81$) indicating that growth rates of mussels younger than 2 years must be higher in high-N estuaries than in low-N estuaries. Growth of young mussels appears to be fostered by increased food availability provided by the increased POM in N-enriched estuaries.

Growth of older mussels (>2 years) is slower in nutrient-enriched environments, and must therefore be affected by factors other than food availability, such as population density. Nutrient loading may lead to increased population density of mussels in an estuary, which may in turn depress growth rates among older mussels. We did not assess density in this study, but past studies have shown that food availability may strongly influence the success of planktotrophic larvae, such as the larvae of mussels (4, 5). If mussel larvae in high-N estuaries have a higher survival rate, increased settlement would lead to increased density, forcing tightly clumped adult mussels to compete for both space and food, and thereby depressing growth rates (7, 8).

This work was supported by funds from a grant from the NSF Research Experience for Undergraduates program.

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Changes in N and C Stable Isotope Signatures of Particulate Organic Matter and Ribbed Mussels in Estuaries Subject to Different Nutrient Loading

Stephanie Yelenik, James McClelland, Noah Feinstein, and Ivan Valiela (Boston University Marine Program, Marine Biological Laboratory)

Geukensia demissa, the ribbed mussel, consumes particulate organic matter (POM) consisting of varying proportions of macrophyte and phytoplankton depending on its location in salt marsh ecosystems (1). Estuaries subject to different nitrogen loads, however, are often dominated by different primary producers (2). Thus, the mix of POM available to *G. demissa* can change not only along gradients within estuaries, but among them. In Waquoit Bay, Massachusetts, increased N loads from anthropogenic activity have resulted in decreased eelgrass (*Zostera marina*) biomass, and increased macroalgal and phytoplankton biomass (2). This study takes advantage of the different N and C stable isotope signatures found in these major producers to examine the sources of particles consumed by *G. demissa* living in estuaries subject to different nitrogen loads.

Ten mussels (average age 6 y) were collected from the same

tidal height within three estuaries receiving high (Childs River), medium (Quashnet River), and low (Sage Lot Pond) nitrogen loads. Collection sites were chosen along the salt marsh bank directly facing the open water to control for effects of location on mussel diet within each site (1). Mussels were kept alive for 24 h to allow guts to clear, then dried and ground into composite samples. POM was collected on ashed Whatman GF/F filters, and isotope analysis of all samples was conducted at Boston University.

POM was sampled in October 1993, July 1994, July 1995, and June 1996; *G. demissa* was sampled only in June 1996. Tissue turnover times of 6-y-old mussels are on the order of 3 years (3), so that *G. demissa* sampled on a single date should reflect the average isotopic signature of foods assimilated during different seasons.

G. demissa and POM $\delta^{15}\text{N}$ show a linear increase across estuaries, with *G. demissa* consistently 3‰ enriched relative to POM (Fig. 1, top). The increase in $\delta^{15}\text{N}$ of POM is consistent with that found for Waquoit Bay macroalgae and plankton responding to increased N loads from wastewater (4). The 3‰ enrichment in *G. demissa* relative to POM is characteristic of fractionation between consumers and their food source (5), indicating that *G. demissa* is a direct consumer of POM.

POM $\delta^{13}\text{C}$ in Sage Lot is higher and more variable than POM in Quashnet or Childs (Fig. 1, middle). The enriched average $\delta^{13}\text{C}$ of POM at Sage Lot looks much like macroalgae, whereas $\delta^{13}\text{C}$ signatures for Childs and Quashnet fall close to a pure phytoplankton signal. POM $\delta^{13}\text{C}$ in Sage Lot, however, reaches values 2‰ higher than those for macroalgae in this estuary, suggesting that another less negative source of POM such as *Z. marina* ($\delta^{13}\text{C} = -7.2\text{‰}$) is contributing to its signal.

Seasonal changes in *Z. marina* biomass may account for the large variability in Sage Lot POM $\delta^{13}\text{C}$ signatures. As biomass of *Z. marina* increases or decreases seasonally, POM $\delta^{13}\text{C}$ signatures at Sage Lot become enriched or depleted (Fig. 1, bottom). Seasonal variability is low in Childs and Quashnet POM $\delta^{13}\text{C}$ because N loading has resulted in loss of *Z. marina* from these estuaries (6).

Unlike the $\delta^{13}\text{C}$ of POM, the $\delta^{15}\text{N}$ of POM is not correlated with changes in *Z. marina* biomass ($P = 0.72$, $r^2 = 0.08$), even

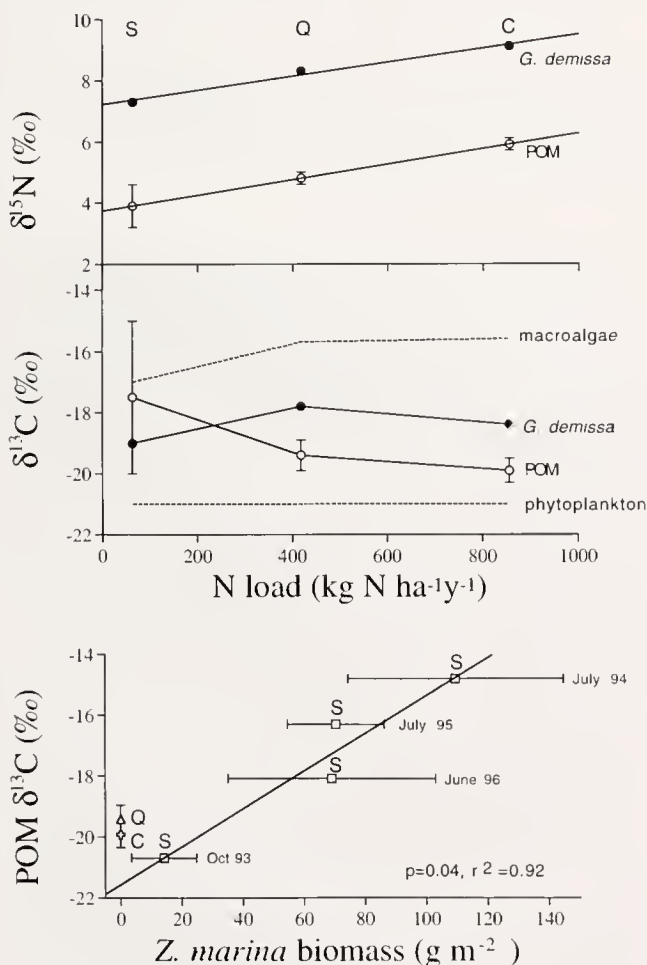


Figure 1. N loading versus $\delta^{15}\text{N}$ (top) and $\delta^{13}\text{C}$ (middle) for *G. demissa* and POM, and *Z. marina* biomass versus POM $\delta^{13}\text{C}$ (bottom) in the Sage Lot Pond (S), Quashnet River (Q), and Childs River (C) estuaries of Waquoit Bay. $\delta^{13}\text{C}$ of macroalgae (average of two most abundant species weighted by biomass contribution in each estuary; McClelland, unpub. data) and Narragansett Bay phytoplankton (7) are plotted in the middle panel for reference. *Z. marina* biomass is from Waquoit Bay Land Margin Ecosystems Research data. Points with error bars are means $\pm$ 1 SD.

though *Z. marina* has a low $\delta^{15}\text{N}$ signature (0.3). The uncoupling of *Z. marina* N and C isotope signals in POM may be the result of the high C:N ratio in *Z. marina* relative to macroalgae and phytoplankton; changes in *Z. marina* biomass should have a greater influence on $\delta^{13}\text{C}$ than on $\delta^{15}\text{N}$ in POM.

Given that consumers are typically enriched in $\delta^{13}\text{C}$ by 1‰ relative to their food sources (5), *Z. marina* cannot be important in the diet of Sage Lot *G. demissa*, even though *Z. marina* makes a notable contribution to POM. Instead, phytoplankton appears to make up the majority of the *G. demissa* diet at Sage Lot, as is the case in Quashnet and Childs (Fig. 1, middle), where *Z. marina* is absent. This similarity indicates that *G. demissa* either selectively ingests or digests specific components of POM. Therefore, even though there are different combinations of primary producers that create the POM in estuaries of Waquoit Bay subject to different N loads, *G. demissa* consistently derives its nutrition from phytoplankton.

This work was supported by a grant from the NSF Research

Experience for Undergraduates program (S. Y., N. F.), the Waquoit Bay Fellowship (J. M.), and the WHOI Sea Grant program (I. V., J. M.).

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Photochemical and Microbial Processing of Dissolved Organic Matter in Streams and Soilwater

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The source and fate of dissolved organic carbon (DOC) is one defining factor for aquatic ecosystems and is a vital link in the global carbon cycle. It is generally thought that most of the organic carbon is processed by microbes, but solar radiation has recently been identified as important in degrading DOC in lakes (1, 2) and in the ocean (3). It has been suggested that light and microbes may work synergistically to oxidize DOC in these natural aquatic systems (4). Our study examined the photochemical and microbial degradation of DOC in a headwater stream system, which represents another important global reservoir of organic carbon.

From a small (50 ha) forested catchment in northern Sweden, we collected water from three sources characterized by different levels of DOC: water from a mire (bog) outlet (high [DOC]), streamwater (intermediate [DOC]), and soilwater (low [DOC]). The samples were filtered at 1.2 μm and incubated in 1-liter glass bottles at 15–20°C for 12 days. Filter-sterilized (0.2 μm) samples were also incubated as an abiotic control. Parallel incubations were carried out in the dark and the light (85 W/m² at 330–800 nm, comparable to incident light in the source catchment).

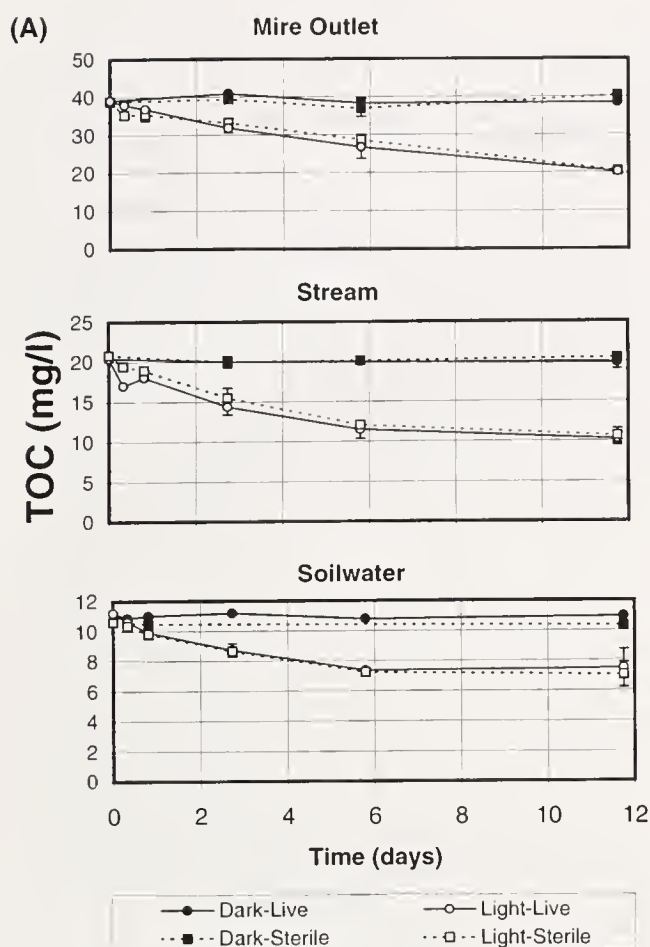
The degradation process was measured as total organic carbon (TOC) concentration (5), and microbial activity was measured as bacterial productivity (³H-leucine incorporation) (6).

Three lines of evidence suggest that the degradation of organic matter in these waters was primarily due to abiotic, photochemical degradation. First, for all treatments, there were no statistical differences in TOC between the live incubations and the sterile-filtered samples. The sterile-filtered incubations had no microbial activity for at least the first 2–5 days, so they functioned as an abiotic control for that time interval. Second, our results showed no significant change (slope within 95% confidence interval of zero) in the TOC concentrations in the dark (Fig. 1A). In the light, however, TOC dropped significantly for all sites over 12 days: from 39.1 to 20.2 mg/l (–48%) in the mire outlet water, from 20.4 to 10.2 mg/l (–50%) in the streamwater, and from 11.2 to 7.5 mg/l (–33%) in the soilwater. Third, bacterial productivity measurements gave estimates of microbial utilization of organic carbon that were small compared to observed decreases in TOC (Fig. 1B).

By integrating bacterial productivity and assuming a 30% efficiency for bacterial assimilation of DOC, we predicted a maximum of 2.0 ± 0.7 mg TOC uptake ($10 \pm 4\%$ of initial TOC) in 12 days for the streamwater light treatment. For all other treatments, estimates of bacterial uptake were even lower compared to the 30%–50% declines observed for TOC in the

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light. Productivity was, however, stimulated in the light treatment for the stream and soilwater sites, suggesting that bacteria utilized the photodegraded organic carbon.

We found that DOC in humic-rich headwaters in northern Sweden was degraded primarily by light rather than by the ambient microbes. The amount of photodegradation was significant and should be included in calculations of carbon flow in

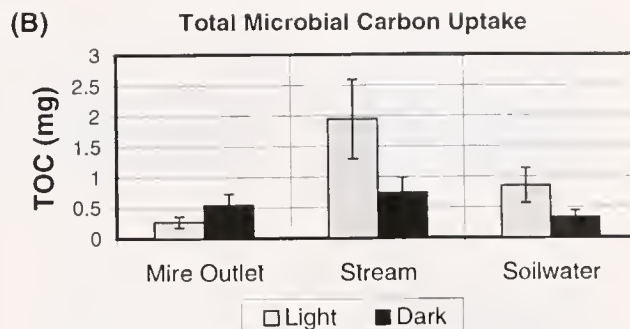


Figure 1. (A) Average concentration of total organic carbon (TOC) for a 12-day incubation of water from three sites, with live (1.2- μ m-filtered)/sterile (0.2- μ m-filtered) and light/dark treatments. TOC was measured by high-temperature combustion on a Shimadzu TOC analyzer (5). (B) Total uptake of organic carbon by microbes in the live treatments throughout the course of the 12-day incubation, as calculated from bacterial productivity measurements.

aquatic ecosystems. Furthermore, photodegradation appeared to enhance bacterial growth, and photo-effects should be accounted for when examining the growth of microbes in the stream water column.

This work was supported by grants from the Swedish Environmental Protection Agency and the Plum Island Sound LMER project (NSF-OCE-9214461). Thanks to Kerstin Kotke for invaluable assistance with sampling and analyses.

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Particulate and Dissolved Nitrogen: A Laboratory Study of Transformations in Groundwater and Estuarine Samples of the Waquoit Bay Estuarine System

Angela F. Dickens, Lori A. Soucy, and Ivan Valiela (Boston University Marine Program, Marine Biological Laboratory)

Estuarine water contains particulate (PN), dissolved organic (DON), nitrate (NO_3^-) and ammonium (NH_4^+) nitrogen. In particular, DON is a major component, making up a considerable proportion of the total nitrogen, but its fate and transformations are poorly understood (1). To quantify short-term interconversions between these forms of nitrogen, we ran labora-

tory incubation experiments of groundwater and estuarine water from the Waquoit Bay watershed-estuary system.

Different estuaries of Waquoit Bay are subject to different nitrogen inputs from their watersheds (2). Sampling from three estuaries allowed study of the effects of different nitrogen loading rates on nitrogen transformations. Stable nitrogen isotopic

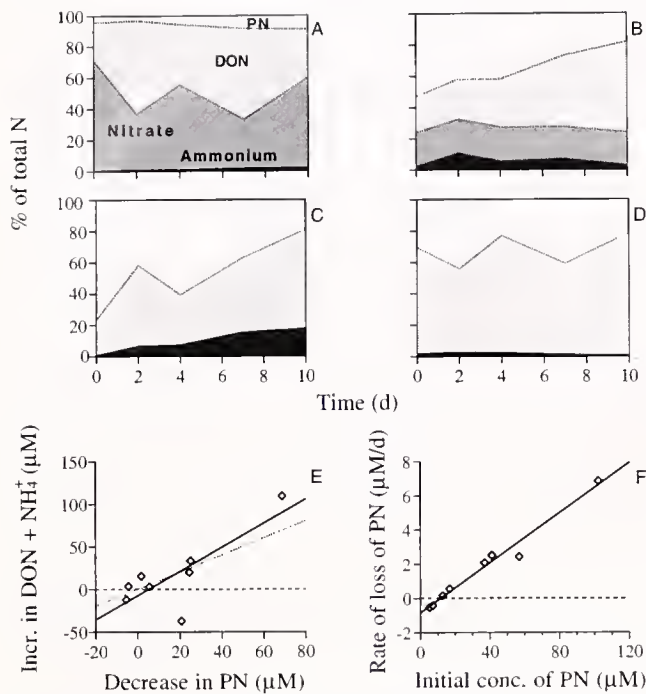


Figure 1. (A–D) Percent composition of samples over the course of the experiment for Childs River (A) groundwater, (B) low salinity water column, (C) mid salinity water column, and (D) high salinity water column. Concentrations of total nitrogen for the samples were: A: 171 μM , B: 82 μM , C: 308 μM , and D: 54 μM . (E) Change in DON plus ammonium versus change in PN concentrations for all three estuaries. The slope of the best-fit line (1.4) is near one (see superimposed dashed 1:1 line). (F) Decrease of PN versus initial PN concentrations for all three estuaries.

analysis has shown that nitrogen from the aquifer is the major source of nitrogen in the estuaries (3). Thus land-derived nitrogen moves down-estuary and is transformed within the Waquoit Bay system.

We collected surface water in three sites from each estuary and used a 253 μm filter to remove consumers. Groundwater was collected with a piezometer. Samples of ground- and estuarine water (400 ml) were incubated in the dark in aerated Erlenmeyer flasks. Flasks were kept in a running seawater bath to hold water temperatures constant. Each flask was sampled after 2, 4, 7, and 10 days. Standard methods were used to determine nutrient concentrations (4, 5).

The concentrations of PN, DON, NH_4^+ , and NO_3^- changed during the incubations. We show only results from Childs

River (Fig. 1A–D) as examples demonstrating the relative interconversions of these nitrogen species. The nitrogen in groundwater showed little evidence of transformations, with no consistent changes in the dominant species, NO_3^- and DON (Fig. 1A). In contrast, the PN in low and mid salinity estuarine water decreased markedly (Fig. 1B and C), and DON increased. In the high salinity reaches (Fig. 1D) the nitrogen species showed little change during the incubations (Fig. 1D).

The gradient between fresh groundwater and salty water (compare Fig. 1A–D) is therefore one in which fairly refractory forms of PN and DON, along with high concentrations of nitrate, are injected into the estuary by groundwater (Fig. 1A). The nitrate is converted to labile PN in the fresher reaches of the estuary (as shown by the shift from nitrate to PN as the dominant species, Fig. 1A–C). By the time the water reaches the salty (25‰) end of the estuary, nitrate is mostly gone, and fairly refractory DON has become the dominant form of nitrogen (Fig. 1D), as it is in this region's coastal seawater (J. McClelland, Boston University Marine Program, pers. comm.).

We can quantitatively examine some of the transformations that occurred during our experiment using the data for all the estuaries (Childs River, Quashnet River, and Sage Lot Pond) (Fig. 1E, F). The increase in DON plus ammonium [the two principal end-products of microbial decomposition of PN (6)] is quantitatively similar to the loss of PN (Fig. 1E). The rate of loss of PN depends on its initial concentration (Fig. 1F); the greater the amount of PN (as a result of increased nitrate loading rates), the greater the amount of PN that can be decomposed. Nitrogen loading therefore provides degradable nitrogen species. These results suggest that increased N inputs from watersheds lead to increased loss of PN and that there is a direct transformation of PN to DON and ammonium.

We thank Ken Foreman for his assistance with the CHN Elemental Analyzer. This work was supported by funds from the NSF Research Experience for Undergraduates program.

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Gas Exchange Rates in the Parker River Estuary, Massachusetts

Stephen Carini (University of Colorado), Nathaniel Weston¹, Charles Hopkinson², Jane Tucker², Anne Giblin², and Joseph Vallino²

Gas exchange rates between natural waters and the atmosphere are an important component of our understanding of the dynamics of biologically active gases. For example, estimates of whole system metabolism must account for the transfer of gas (O_2) between the atmosphere and water. Previous studies in estuaries have relied on dome measurements to estimate O_2 exchange rates (1, 2). In this study we measured gas exchange rates using sulfur hexafluoride (SF_6) as a tracer. SF_6 is well suited for this application because it is chemically and biologically nonreactive, occurs at low background levels, and extremely low concentrations are readily detectable (3). The gas exchange coefficient, k , calculated for SF_6 can be related to the exchange coefficient for other gases (4).

We injected ~ 0.004 moles of SF_6 into the Parker River estuary, Newbury Massachusetts, and monitored the evasion of the gas over time by the decrease in its total mass. The tracer was allowed to mix for one tidal cycle and we then sampled SF_6 concentration in the water at each of seven successive high tides. Surface water samples were drawn into 100-ml glass syringes, transported, submerged in river water, to a field laboratory, and analyzed within 6 h using gas chromatography with electron capture detection. Windspeed and precipitation data were recorded continuously. Several previous surveys were used to determine cross-sectional areas along a 10-km stretch of the estuary. The total mass of SF_6 in the estuary was calculated by integrating concentration and water volume by estuarine distance.

The distribution of the tracer changed over time in relation to processes controlling mixing and loss to the atmosphere (Fig. 1a). After the initial tidal cycle the tracer plume measured 5.2 km in length, and the distribution was gaussian. The exchange coefficient, k , and mass are related by the function $k = \ln(M/M_0)h/t$ where M is the measured mass of SF_6 in the estuary, M_0 is the previously measured mass of SF_6 , h is the depth, and t is the time between samplings. Calculated values for k_{SF_6} range from 1.1–6.2 $cm \cdot h^{-1}$. Fluctuations in k are well correlated with wind velocity [in agreement with previous studies (4, 5)] and with precipitation (Fig. 1b). These values are lower than those predicted from wind relations established from dome studies (Fig. 1c). For estuarine systems with complicated geometry (e.g., channel longitudinal direction, marsh grass, and high tidal range), direct measurement of SF_6 evasion may be a more accurate determination of gas exchange rates.

The importance of gas exchange as a process influencing the determination of system metabolism was determined by applying our measured gas exchange coefficient for SF_6 to the calculation of O_2 gas exchange. System respiration was calculated by mass loss of dissolved oxygen between dusk and dawn (Fig. 1d) and corrected for gas exchange with the atmosphere. The gas transfer velocities of O_2 and SF_6 are related by the function k_{O_2}

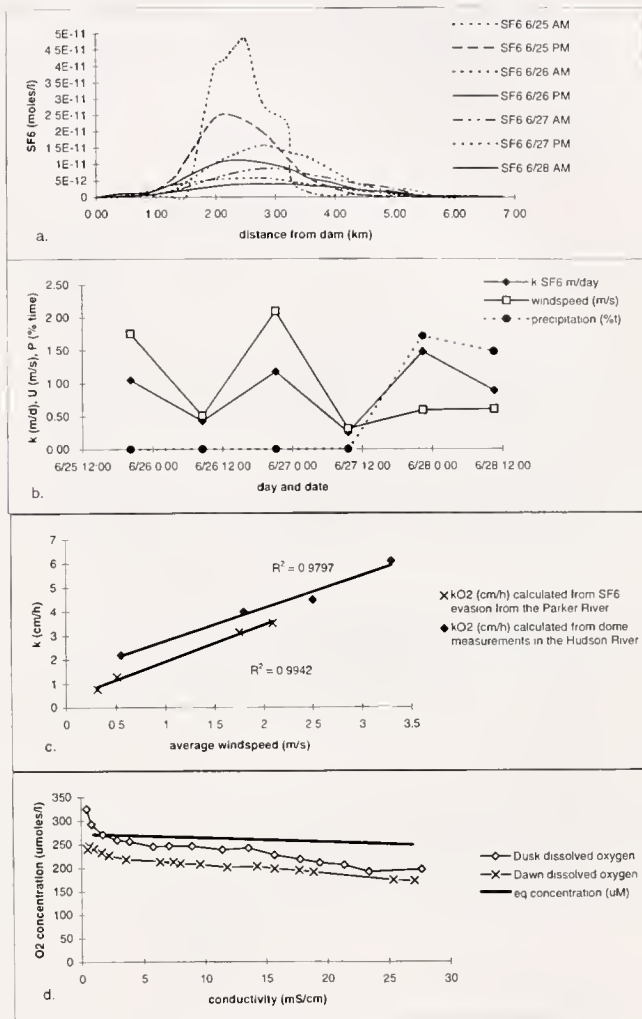


Figure 1. (a) Concentration and distribution of SF_6 measured from the Parker Dam. (b) k as a function of windspeed (U) and precipitation (P). $k = 2.15547U + 8.099P + 0.04785$ with a total model R^2 of 88.7 ($P = .037$). (c) k_{O_2} measured by the dome method in the Hudson River (1) and by measurement of SF_6 evasion from the Parker River Estuary. (d) Dawn and dusk dissolved oxygen concentrations plotted against conductivity.

$= k_{SF_6} (Sc_{SF_6})^n / (Sc_{O_2})^n$ where Sc is the Schmidt number and n is assumed to be $-2/3$ (4). The resulting k for O_2 was 1.28 $cm \cdot h^{-1}$ and a net influx of O_2 was added to the total mass loss of oxygen. This resulted in a calculated respiration rate of 208 $mmoles O_2 \cdot m^{-2} \cdot d^{-1}$. Although the correction for gas exchange during the period of this study was less than 0.15%, under different environmental conditions, such as higher concentration gradients or windspeeds, corrections would be substantially greater.

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This research was partially supported by grants from WHOI Sea Grant, the Plum Island Sound LMER, the Sweetwater Trust, and the NSF-REU program. We gratefully acknowledge the help of Jim Ledwell, who graciously provided both his expertise and equipment.

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Estimating Denitrification in Sediments of the Parker River Estuary, Massachusetts

Nathaniel Weston (Hampshire College), Stephen Carini¹, Anne Giblin², Gary Banta³, Charles Hopkinson², and Jane Tucker²

Nitrogen cycling plays a major role in determining the level and pattern of estuarine productivity, because most coastal ecosystems are nitrogen limited (1, 2). The fate of inorganic nitrogen entering an estuary is key to understanding estuarine nitrogen dynamics. Denitrification, an anaerobic respiration process in which nitrate is reduced to N₂ gas by bacteria (1, 2, 3), is an important nitrogen sink in estuarine systems. Nitrate

for denitrification in the sediments comes from two sources: diffusion of NO₃⁻ into the sediment from the water column (direct denitrification), or NO₃⁻ produced from the oxidation of ammonium (NH₄⁺) released by the degradation of organic matter (coupled denitrification) (1, 4, 5).

The purpose of this study was to investigate sediment denitrification at an oligohaline site in the Parker River Estuary, Massachusetts. Our objectives were to compare (1) three methods of estimating coupled denitrification, (2) rates of denitrification with several NO₃⁻ concentrations in the overlying water and (3) denitrification rates in intertidal and subtidal sediments.

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Table 1

Sediment-water fluxes of DIC, O₂, NH₄⁺, and NO₃⁻, sediment NH₄⁺ production, and estimates of denitrification for the Parker River Estuary (mean ± standard error). Fluxes are in mmol·m⁻²·d⁻¹, and denitrification estimates are in mmol N·m⁻²·d⁻¹. Negative fluxes are directed into the sediment. Ambient nitrate concentrations were ~9 μM.

Treatment: Site:	+0 μM NO ₃ ^{-a} Intertidal Flux ± SE	+0 μM NO ₃ ^{-a} Subtidal Flux ± SE	+5 μM NO ₃ ^{-a} Subtidal Flux ± SE	+25 μM NO ₃ ^{-b} Subtidal Flux ± SE
NH ₄ ⁺ flux	2.64 ± 0.41	2.02 ± 0.10	3.69 ± 1.35	2.32 ± 0.37
O ₂ flux	-35.53 ± 1.01	-27.01 ± 0.54	-44.47 ± 10.41	-38.47 ± 4.90
O ₂ :N ratio	13.86 ± 6.01	13.40 ± 0.74	12.74 ± 2.40	17.30 ± 4.92
Coupled denitrification	2.73 ± 0.56	2.05 ± 0.18	3.03 ± 0.22	3.49 ± 1.03
DIC flux	65.71 ± 5.30	18.62 ± 0.55	32.14 ± 4.11	23.55 ± 6.98
C:N ratio	25.85 ± 6.01	9.24 ± 0.74	9.59 ± 2.40	9.92 ± 1.53
Coupled denitrification	7.28 ± 1.21	0.79 ± 0.19	1.16 ± 0.73	1.24 ± 0.68
NH ₄ ⁺ production ^c	8.48 ± 1.94	5.37 ± 1.95	5.37 ± 1.95	5.37 ± 1.95
Coupled denitrification	5.84 ± 1.98	3.35 ± 1.95	1.69 ± 2.37	3.05 ± 1.99
NO ₃ ⁻ flux	-0.48 ± 0.14	0.00 ± 0.04	-0.87 ± 0.49	-2.38 ± 0.40
Direct denitrification	0.48 ± 0.14	0.00 ± 0.04	0.87 ± 0.49	2.38 ± 0.40
Total denitrification ^d	3.20–7.76	0.79–3.35	2.03–3.89	3.62–5.87
% direct denitrification ^e	6–15%	0%	22–43%	41–66%
% N denitrified ^f	50–73%	50–62%	26–47%	32–59%

Notes: ^an = 2; ^bn = 3; ^cn = 9; ^dcalculated as a range for coupled + direct; ^ecalculated as a range of percent of total denitrification; ^fcalculated as a range of percent remineralized N denitrified.

Intact sediment cores were collected from a subtidal and an intertidal site and incubated to determine exchange rates of oxygen (O_2), dissolved inorganic carbon (DIC), NH_4^+ , and NO_3^- between the sediment and the overlying water. NH_4^+ production in the sediment was determined by the increase of NH_4^+ (6, 7) in separate cores incubated anaerobically. Direct denitrification was measured by NO_3^- uptake from the overlying water. Coupled denitrification was estimated by comparing measured NH_4^+ release to several expected NH_4^+ release rates calculated from estimates of sediment metabolism based on O_2 uptake, DIC release, and anaerobic NH_4^+ production (7, 8). The expected NH_4^+ release was calculated by assuming C:N and O_2 :N ratios of 6.625, based on the Redfield ratio (7, 9).

Estimated rates of denitrification ranged from 0.79 to 7.76 $mmoles\ N \cdot m^{-2} \cdot d^{-1}$ (Table I), which is in good agreement with other estuarine studies (1, 5, 10). Overall, the various methods did not differ significantly ($P > 0.05$), although for subtidal cores, estimates based on DIC release were lower than estimates based on both NH_4^+ production and O_2 consumption ($P < 0.05$). For the intertidal cores, estimates based on O_2 consumption were lower than estimates from NH_4^+ production ($P < 0.05$). Between 25% and 75% of the nitrogen regenerated in the sediments was estimated to have been denitrified (Table I), indicating that denitrification is a significant sink for nitrogen in Parker River Estuary.

Coupled denitrification did not significantly change with increasing NO_3^- concentration ($P > 0.05$). Direct denitrification, however, did increase with nitrate addition ($Flux = 0.0841[NO_3^-] - 0.46$; $P < 0.05$). Whereas NH_4^+ oxidation was the major source of nitrate for denitrification under our ambient conditions (1), denitrifying bacteria were able to take advantage of higher nitrate concentrations.

The estimates of denitrification based on DIC release and

NH_4^+ production were higher at the intertidal site than the subtidal site ($P < 0.05$), and there was evidence of direct denitrification in the intertidal cores at ambient NO_3^- levels (Table I). Higher rates of organic matter remineralization indicate higher rates of metabolism at the intertidal site during our investigation. Oxygen uptake did not reflect the higher rate of metabolism (Table I), possibly due to the storage of reduced sulfur compounds from anaerobic sulfate reduction (7). The differences between intertidal-subtidal metabolism and oxidation-reduction capacity warrant further research.

This research was partially supported by grants from WHOI Sea Grant, the Plum Island Sound LMER, the Sweetwater Trust, and the Woods Hole Marine Science Consortium.

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Number 3

THE BIOLOGICAL BULLETIN



DECEMBER, 1996

Published by the Marine Biological Laboratory

THE BIOLOGICAL BULLETIN

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DECEMBER, 1996

Printed and Issued by
LANCASTER PRESS, Inc.

3575 HEMPLAND ROAD
LANCASTER, PA

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is published six times a year by the Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543.

Subscriptions and similar matter should be addressed to Subscription Manager, THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543. For 1997, a lower rate is available to individual subscribers (as distinguished from libraries and institutions). Single numbers: \$40 for libraries; \$20 for individuals. Subscription per volume (three issues): \$97.50 for libraries; \$50 for individuals. Subscription per year (six issues, two volumes): \$195 for libraries; \$100 for individuals.

Communications relative to manuscripts should be sent to Michael J. Greenberg, Editor-in-Chief, or Pamela L. Clapp, Managing Editor, at the Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543. Telephone: (508) 289-7428. FAX: 508-457-1924. E-mail: pclapp@mbi.edu.

THE BIOLOGICAL BULLETIN is indexed in bibliographic services including *Index Medicus* and MEDLINE, *Chemical Abstracts*, *Current Contents*, and *CABS (Current Awareness in Biological Sciences)*.

Printed on acid free paper,
effective with Volume 180, Issue 1, 1991.

POSTMASTER: Send address changes to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543.

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Periodicals postage paid at Woods Hole, MA, and additional mailing offices.
ISSN 0006-3185

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All-or-None Contraction and Sodium Channels in a Subset of Circular Muscle Fibers of Squid Mantle

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Motor function in squid (Loligo) mantle reflects the highly coordinated activity of two motor pathways associated with giant and non-giant motor axons that respectively produce all-or-none and graded contractions in mantle muscle. Whereas both types of axons innervate circular mantle muscle fibers, precise nerve-muscle relationships remain unclear. Are squid like most invertebrates, in which single muscle fibers receive dual innervation from giant and non-giant motor axons, or is squid mantle configured more like vertebrates, in which parallel motor axon systems innervate distinct fast and slow muscle fibers? In this report, we describe giant and non-giant motor pathways that appear to control different pools of circular muscle fibers in squid. A subset of circular muscle fibers possesses large Na currents, and these fibers are proposed to employ Na-dependent action potentials to produce fast, all-or-none muscle twitches associated with giant axon stimulation.

Fast and slow motor systems with diverse properties occur in many taxa (1). Studies on arthropods and chordates have revealed remarkable differences in basic neuromuscular organization, but comparative knowledge of other groups is relatively limited. Dual motor pathways also exist in cephalopod molluscs (2). In many squid species, giant motor axons innervate extensive mantle fields composed of small-diameter, circular muscle fibers, and a single axonal impulse generates a powerful all-or-none twitch (3). A parallel set of non-giant motor axons acts to generate graded mantle contractions with repetitive stimulation (4, 5). Concerted recruitment of these two systems plays an important role in controlling jet-pro-

pelled escape responses (6). Cephalopod muscle fibers are very small, and patterns of innervation for the two motor systems have not been elucidated.

Relative contributions to the mantle twitch made by these two motor pathways in squid can be evaluated by measuring contractile force due to circular muscle fibers in response to selective stimulation of giant and non-giant motor axons. In Figure 1A, a single shock excited only non-giant axons, and a stronger shock exceeded threshold for giant axon stimulation (Fig. 1B). Repetitive non-giant activity was stimulated by a brief, weak tetanus, and the resulting contractile response (Fig. 1C) is directly compared to the giant-driven response in Figure 1D (trace C versus B). The algebraic sum of traces B and C (B + C, dotted trace) is smaller than the response generated by a burst of strong shocks that excited both systems (thick trace) but is quite similar in time course. Thus, outputs of the two motor systems are roughly additive and combine to produce a powerful mantle contraction with a fast rise time.

Analysis of the relevant circular muscle elements has been limited, however. Three concentric layers of circular muscle fibers exist in squid mantle, with two superficial layers of mitochondria-rich fibers surrounding a much thicker central zone of smaller diameter, glycolytic fibers (7, 8). Based on these findings, it is believed that the central zone, innervated by the giant axons, generates escape jetting and that the superficial layers control slower, sustained swimming (2). Several observations, however, suggest that this division of labor is too simple. First, the superficial layers constitute no more than 20% (7, 8) of the total circular muscle mass, and it therefore seems unlikely that this small fraction could generate a twitch as large as that produced by stimulation of the giant axon (Fig. 1). Second, behavioral experiments indicate that squid can swim at variable velocities up to

Received 7 August 1996; accepted 21 August 1996.

Abbreviations: G_{Na} , Na conductance density; GFL, giant fiber lobe; I_K , K current; I_{Na} , Na current.

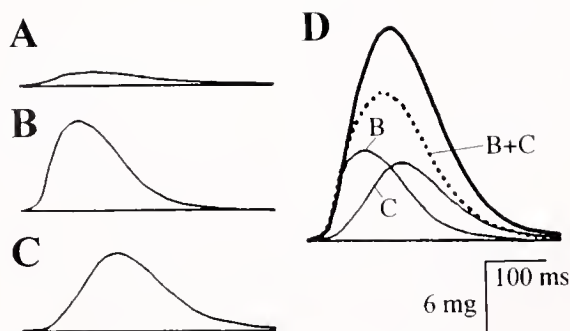


Figure 1. Non-giant and giant motor axons contribute approximately equally and additively to the twitch of circular muscle fibers. The nerve-muscle preparation consisted of a stellate ganglion from a juvenile *Loligo opalescens* connected by stellar nerves to a sector of mantle cut parallel to the circular muscle bands and attached to a force transducer (BG-10GM, Kulite Inc., Leonia, NJ). Motor axon stimulation was effected with a coaxial, bipolar electrode. (A) A single 3 V shock excited only non-giant motor axons. (B) A single 5 V shock exceeded threshold for giant axon excitation. (C) Summed response due to stimulation of non-giant motor axons with five 3 V shocks at 50 Hz. (D) Superposition of the records in panels B and C along with their algebraic summation ($B + C$, dotted trace) is compared with the responses to five 5 V shocks at 50 Hz which excite giant and non-giant motor systems. External solution was artificial seawater. $T = 16^\circ\text{C}$.

and including those characteristic of escape jetting (9). Third, giant and non-giant axon-driven responses can contribute nearly equally to escape jetting (6).

As a first step towards definition of the relevant neuromuscular relationships in squid muscle, we have attempted to identify a muscle fiber type with electrical properties suggestive of all-or-none excitability, and which therefore is likely to be associated with the giant axon system. Whole-cell voltage clamp recordings from dissociated mantle muscle fibers were carried out using a Na-containing internal solution that blocks K channels (10). Figure 2A illustrates a muscle fiber shortly after achieving the whole-cell configuration and after repetitive pulsing (inset), which caused irreversible contraction. This phenomenon reliably yielded well-clamped cells, and all data in this paper were similarly obtained. Transient inward current recorded from this muscle fiber at 0 mV (Fig. 2B_i) is eliminated by an inactivating prepulse (+pre; 50 ms to -30 mV) or by tetrodotoxin (+TTX; 500 nM). Figure 2B_{ii} illustrates analogous records from a cultured giant fiber lobe (GFL) neuron in which Na currents (I_{Na}) have been studied in detail (10). A family of prepulse-sensitive current traces from this muscle fiber (Fig. 2C) indicates a reversal potential close to the expected Nernst potential for Na ($\sim +20$ mV).

Taken together, these features demonstrate that this muscle fiber displays I_{Na} much like that in GFL neurons or the giant axon. This is not true for all mantle muscle fibers, however. Current records at 0 mV are illustrated

for three different muscle cells in Figures 3A–C. These recordings were made with a high K internal solution (11) in order to assay both inward I_{Na} and outward K currents (I_{K}). Long fibers of very thin diameter (Fig. 3A) exhibited robust I_{Na} , whereas larger diameter fibers (Fig. 3B) typically showed no or very little I_{Na} . Regardless of the I_{Na} level, all fibers expressed substantial I_{K} . Multipolar muscle cells (Fig. 3C) were also regularly encountered, and these too showed no I_{Na} . With the internal solution designed to eliminate I_{K} , all cell types displayed small, non-inactivating inward currents with properties similar to Ca currents in GFL neurons (12).

To more accurately compare Na channel density in different muscle cells, maximum Na conductance for each cell was computed from the final slope of the peak

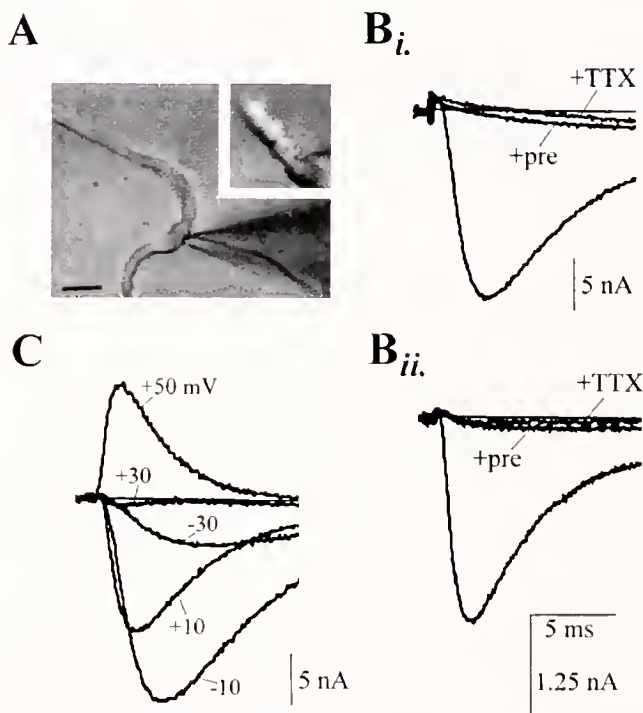


Figure 2. I_{Na} in a muscle fiber from squid mantle. Skinned mantles from juvenile squid (55–57 days old) were cut into strips parallel to circular muscle bands in Ca-free seawater and treated with protease (Type XIV, Sigma) for 40 min at 17°C . Strips were triturated through a micropipette to dissociate muscle fibers, which were then maintained up to one day at 17°C in a low-Ca medium (adapted from (15), external $\text{Ca} = 2\text{--}3$ mM). (A) Video prints during a whole-cell recording (see text for details; scale bar for this and all video prints = $20\text{ }\mu\text{m}$). (B_i) Inward current at 0 mV (holding potential = -80 mV for this and all other figures) from the fiber in panel A without (large amplitude trace) and with either a depolarizing prepulse (+pre) or exposure to 500 nM tetrodotoxin (+TTX) in the external solution. (B_{ii}) Records obtained in a GFL neuron (6 days in culture) using the same apparatus and under identical conditions as used for panel B_i. (C) A family of I_{Na} currents (obtained by subtraction of records taken with and without an inactivating prepulse) recorded at the indicated voltages from the muscle fiber of panel A. External solution was culture medium. $T = 10\text{--}12^\circ\text{C}$.

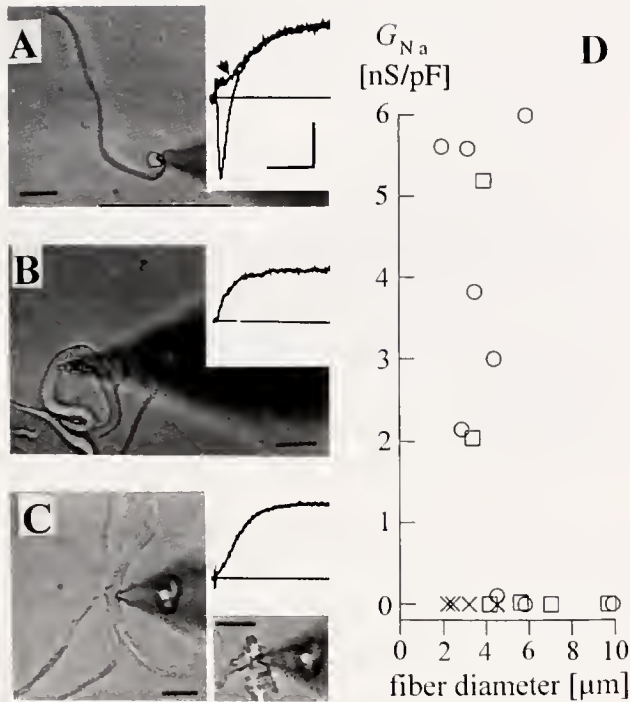


Figure 3. Correlation of Na channel expression and muscle fiber type. All data were derived as in Fig. 2. (A–C) Video prints of three different muscle cells taken shortly after achieving the whole-cell configuration and records of membrane current at 0 mV attained after contracture (inset in Fig. 3C). Currents in Fig. 3A are shown with and without a prepulse to inactivate I_{Na} . I_{Na} was not detectable in the fibers shown in panels B or C (for all current traces, vertical scalebar = 125 pA/pF, horizontal scalebar = 5 ms). (D) Specific Na conductance (G_{Na} ; derived as described in text) is shown plotted versus muscle cell diameter and type (see text for details). Diameter measurements were made from digitized video frames using image analysis software (NIH-Image 1.60), and mean diameter was calculated from 10 measurements equally spaced along the length of the fiber.

$I_{\text{Na}} - V$ relation, and these values were normalized by input capacity (G_{Na} ; nS/pF). These data are plotted versus diameter in Fig. 3D for fibers $< 120 \mu\text{m}$ (□) and $> 120 \mu\text{m}$ (○) in length, and for multipolar-cell arms (×). The approximate thickness of the mantle musculature is $120 \mu\text{m}$ (equal to the length of radial muscle fibers) in a squid of the size used for the dissociations (unpub. data). Fibers longer than this value are thus likely to be derived from circular muscle. Seven out of eight fibers showing significant G_{Na} lie in the $2\text{--}5 \mu\text{m}$ range, and most of these are $> 120 \mu\text{m}$ long. All fibers lacking G_{Na} are $> 4 \mu\text{m}$ diameter, and half of these are $> 120 \mu\text{m}$ long.

Thus, on the basis of the level of G_{Na} , we conclude that two classes of circular muscle fibers exist, and there appear to be two corresponding size classes. Presumably the small, Na-channel-rich fibers are central circular fibers ($3.3 \pm 0.1 \mu\text{m}$ diameter, mean $\pm$ SEM based on unpublished electron microscopic analysis) associated with

the giant axon system, but at present we have no direct evidence. Identification of long fibers devoid of Na channels is less certain. These fibers could either be superficial fibers ($4.8 \pm 0.2 \mu\text{m}$ diameter), or they could represent a distinct type of central fiber associated with the non-giant motor system that contributes to escape jetting. Although anatomical studies have not revealed two types of central circular fibers, existence of a central fiber type showing graded excitability is suggested by the relatively large contribution conferred by repetitive stimulation of non-giant motor axons (Fig. 1C).

Multipolar cells also appear to lack Na-channel-based excitability. This unusual cell type has not been previously detected in anatomical studies, but the small diameter and overall length of the processes would be consistent with the idea that such cells represent radial muscle fibers ($2.7 \pm 0.2 \mu\text{m}$ diameter). Future studies will be required to test this hypothesis. Graded excitability in this type of muscle fiber would fit with the role played by radial fibers *in vivo* (13).

Results in this paper demonstrate the existence of large Na currents in circular muscle fibers of squid mantle, and we propose that these currents provide the basis for the all-or-none contractile response to a single action potential in the giant motor axons. Na-channel-based excitability in invertebrate muscle fibers was previously reported to exist only in the phylum *Chaetognatha* (comprising the “arrow worms”, ref. 14). Theories concerning the evolutionary origin of Na channels in muscle fibers of deuterostome versus protostome invertebrates must also take the data on squid described in this study into account. Clearly, application of patch-voltage clamp techniques to muscle fibers from a wide variety of invertebrate phyla would be a valuable contribution to comparative muscle physiology and might provide insights of evolutionary significance.

Acknowledgment

This work was supported by grants from the National Institutes of Health, the National Science Foundation, the Office of Naval Research, and the Ford Foundation. We are grateful to Gilbert van Dykhuizen (Monterey Bay Aquarium) for providing juvenile squid.

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The Peptide pQFYRFamide Is Encoded on the FMRFamide Precursor of the Snail *Helix aspersa* but Does Not Activate the FMRFamide-Gated Sodium Current

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Abstract. The complete sequence of the FMRFamide precursor cDNA from *Helix aspersa* is reported here. Since the 5' end of this cDNA is identical to that of the precursor that encodes the heptapeptide analogs of FLRFamide, the two transcripts are probably derived by alternative RNA splicing. A novel pentapeptide, Glp-Phe-Tyr-Arg-Phe-NH₂ (pQFYRFamide), predicted from the cDNA sequence, was purified from extracts of *H. aspersa* ganglia and identified by mass spectroscopy. Partial gene sequences for the FMRFamide precursors of two closely related pulmonate species—*Cepaea nemoralis* and *Polydortes acutangula*—were also determined and compared with the cDNA sequence of *H. aspersa* and a partial gene sequence previously determined from *H. pomatia*. Not only are the FMRFamide-related sequences and proteolytic processing sites conserved, but the linear arrangement of these landmarks is also retained. Synthetic pQFYRFamide has some effects on the isolated heart and on neuronal potassium currents in *H. aspersa* that are similar to those of FMRFamide, but it does not activate the neuronal FMRFamide-gated sodium channel.

Introduction

The tetrapeptide FMRFamide (Price and Greenberg, 1977) is undoubtedly present in all molluscs (Greenberg and Price, 1992), but the mRNA encoding the FMRFamide precursor has only been completely de-

scribed from an opisthobranch, *Aplysia californica* (Schaefer *et al.*, 1985; Taussig and Scheller, 1986), and a pulmonate, *Lymnaea stagnalis* (Linacre *et al.*, 1990). Although only a few stretches of amino acid sequence are common to the precursors of these two species [*i.e.*, a single copy of FLRFamide, a sequence SEEPLYRKRRS which includes a tetrabasic proteolytic processing site, and the multiple copies of FMRFamide], the general organization of these two precursors is similar (see Greenberg and Price, 1992).

Pulmonate snails, but not other molluscs, also contain heptapeptides of the form XDPFLRFamide (X = N, S, G, pQ) (Price *et al.*, 1990). These heptapeptide analogs of FLRFamide are encoded on a different precursor from FMRFamide (Saunders *et al.*, 1991; Lutz *et al.*, 1992) and are expressed in different neuronal populations (Bright *et al.*, 1993; Cottrell *et al.*, 1992) in both *Lymnaea stagnalis* and *Helix aspersa*. In *L. stagnalis*, the two precursors are alternatively spliced variants of a single gene which share the same first exon (Saunders *et al.*, 1992). Whether a similar alternative splicing mechanism occurs in *H. aspersa* is unclear because only a partial cDNA encoding the tetrapeptides had been previously isolated (Lutz *et al.*, 1992), notwithstanding that the cDNA encoding the heptapeptide precursor in *H. aspersa* has been fully sequenced (Cottrell *et al.*, 1994).

Because conservation of structure is often associated with functionally important regions of a protein, and because the FMRFamide precursor from only one other pulmonate is fully known, we have determined the complete sequence of the FMRFamide precursor in *H.*

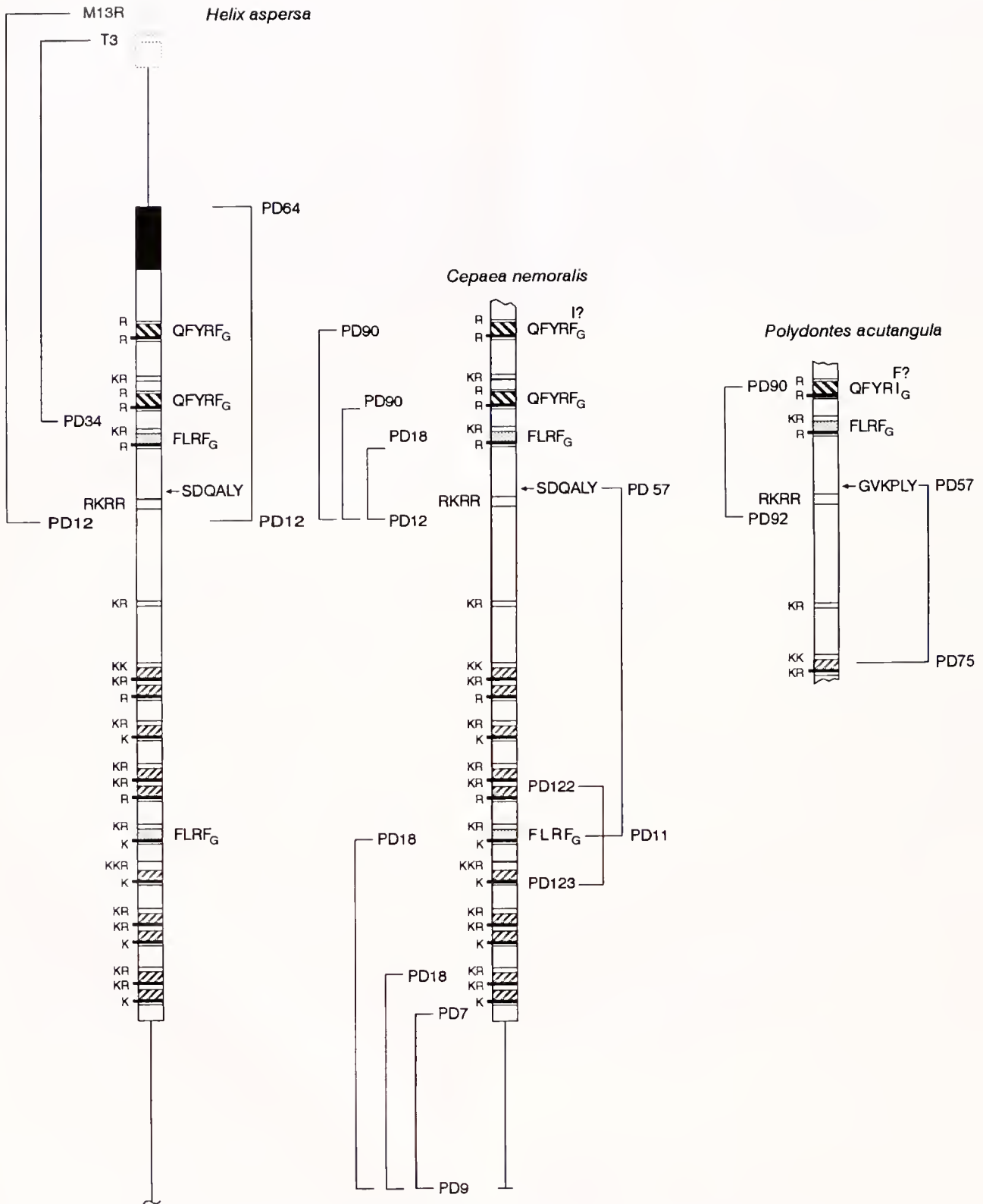


Figure 1. Composite schematic representation of the complete FMRFamide precursor of *Helix aspersa* (GenBank L20768) and precursor fragments from *Cepaea nemoralis* (GenBank U02488) and *Polydotes acutangula*. The noncoding regions are shown as vertical lines and the coding regions as columns. The columns are positioned so that the tetrabasic sequences (RKRR) common to all are horizontally aligned.

aspersa, showing in the process that alternative splicing occurs in this species as well as in *L. stagnalis*. We have also broadened the comparison by examining two additional pulmonate snail species and have established, thereby, landmark sequences that seem to be common features of the FMRFamide precursors of pulmonate and opisthobranch molluscs.

The resulting sequences have revealed a putative novel peptide (pQFYRFamide) that has now been purified from extracts. The pharmacology of FMRFamide-related peptides has already been studied extensively in *H. aspersa*, and we know that the heptapeptide analogs of FLRFamide have some biological actions on peripheral organs and neurons that are distinct from those of FMRFamide and FLRFamide (Lehman and Greenberg, 1987; Cottrell and Davies, 1987). We have therefore surveyed the pharmacology of synthetic pQFYRFamide and report here that, although this pentapeptide has some of the same actions as FMRFamide on hearts and neurons of *H. aspersa*, it has no effect on the neuronal sodium channel that is directly gated by FMRFamide (Cottrell *et al.*, 1990; Green *et al.*, 1994).

Materials and Methods

Animals

Three species of snails from the pulmonate order Stylommatophora, superfamily Helicoidae, were used in this study. Two species, *Helix aspersa* and *Cepaea nemoralis*, are members of the family Helicidae and the third, *Polydonte acutangula*, is in the family Camaeniidae. Note that *Lymnaea stagnalis*, another frequently studied pulmonate, is in the order Basommatophora.

Those specimens of *H. aspersa* used in the purification of pQFYRFamide and in the pharmacological testing of that peptide on snail hearts were collected in Fullerton, California, and shipped to St. Augustine, Florida, by Robert A. Koch. The cDNA library was made from *H. aspersa* collected in St. Andrews, Scotland, and these animals were also used in electrophysiological experiments. Specimens of *Cepaea* were collected in Bellmore, New York, whereas *Polydonte* were obtained in El Yunque rain forest in Puerto Rico.

Library construction

RNA was isolated from clusters of neurons located in the parietal ganglia of *H. aspersa*. These clusters consist almost entirely of neurons immunoreactive to FMRFamide peptides (Cottrell *et al.*, 1992). A directional cDNA library was constructed using the Lambda-Zap kit (Stratagene).

PCR amplifications

Preparation of template DNA. Aliquots (typically 5 μ l, but always less than 10 μ l) of the amplified cDNA library were used directly in the PCR. For amplification of genomic DNA from *Cepaea* or *Polydonte*, a small piece of tissue (typically 1 ganglionic ring, 10 mg) was heated for 10 min at 95°C in about a 5-fold excess of 100 mM NaOH (50 μ l for a ganglion) and was then centrifuged for 5 min at maximum speed in an Eppendorf centrifuge. The supernatant was transferred to a clean tube and diluted 10-fold with water. Aliquots (0.3 to 5 μ l) were used for PCR.

Standard amplification. All of the PCR components, except the DNA polymerase, were assembled in a total volume of 80 μ l in a 0.5-ml tube and overlaid with mineral oil. The tube was heated to 95°C for 10 min, cooled to 80°C, the DNA polymerase (Taq, Promega, 2.5 U in 20 μ l) added through the oil, and the PCR cycles (Perkin Elmer Cetus thermal cycler) started (30 to 40 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C). The PCR reaction mixture contained buffer (Promega: 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, pH 9.0 at 25°C), 1.5 mM Mg, 200 μ M of each dNTP (Pharmacia), template and primers (20 to 200 μ mol). An aliquot (10 μ l) was run on an agarose gel, and reactions that showed products of the expected size were purified for cloning as follows. First, the aqueous phase containing the PCR product was removed from under the oil layer and rolled around on a sheet of Parafilm to remove residual oil droplets (Whitehouse and Spears, 1991). The DNA was then precipitated by the addition of an equal volume of 5 M ammonium acetate followed by two volumes of isopropanol (storage overnight at -20°C). The precipitate was then washed with 70% ethanol, dried in a Speed-Vac, and resuspended in 10 μ l water. Aliquots (1-7 μ l) were

The six amino acid residues just upstream of the tetrabasic sites are also labeled. The positions of individual peptides are stippled or hatched; the copies of FMRFamide are not labeled. Putative, amide-donating, glycine residues are shown as dark lines extending to the left of the column. Thin cross-lines delimit the boundaries of putative, basic processing sites. The signal sequence of the *H. aspersa* precursor is shown in solid black. The segments that were amplified by PCR, then cloned and sequenced, are indicated by brackets labeled with the primers used. Alternative amino acid residues encoded by degenerate PCR primers are shown above the most likely sequence and are followed by question marks. The vector region expected to adjoin the cloned *H. aspersa* sequence is shown with dotted lines. The *H. aspersa* 3' noncoding region (see Lutz *et al.*, 1992) extends further than shown (indicated by a tilde).

ligated into the pGEM-T vector (Promega), which was then transformed into *E. coli* (JM109). Colonies were analyzed by PCR with primers taken from either side of the cloning site. Clones containing an insert of the appropriate size were sequenced with Sequenase (US Biochemicals, Cleveland, Ohio) kits.

Nested PCR. For the initial amplification, 5 μ l from the library was used as the template for a pair of primers. [For an example, consider M13R and PD12; see Fig. 1 for location.] From the initial reaction product, 0.1% to 1% was used as template for the secondary amplifications with two additional primers chosen from the targeted PCR product, and only 25 cycles of amplification were done. [Continuing the example, see T3 and PD34 in Fig. 1.]

Primers. The following oligonucleotide primers were synthesized by the DNA synthesis facility of the University of Florida Interdisciplinary Center for Biotechnology Research. Seven of these primer sequences were taken directly from known DNA sequences: PD7, PD9, and PD12 are from the FMRFamide-encoding clone HF1 (Lutz *et al.*, 1992); PD64 is from the hepta-peptide-encoding clone HF4 (also Lutz *et al.*, 1992); M13 rev and T3 promoter are from near the insertion site of the plasmid cloning vector Bluescript (Stratagene); and PD92 is from the first FMRFamide-encoding PCR product obtained from *Polydectes*. The remaining eight primers were designed from amino acid sequences rather than DNA sequences and are degenerate; the mixed bases are parenthetical in the following list. Inosine (I) was used in positions where all four bases could encode the desired amino acid. Some primers to repeated sequences were designed to be only partially degenerate so that they would *not* match every repeat exactly. For example, PD11, an antisense primer to some DNA sequences encoding RFMRFGK (R or S) exactly matches only one repeat in the *Cepaea* FMRFamide gene. The primer binding sites for all of these oligonucleotides are shown in Figure 1.

PD7 (sense):	CGTGGGGATTGAGAAACATCA-TGA
PD9 (anti-sense):	TAGCGCTAGAACCTACTACCGAT
PD11 (anti-sense):	CTTTTCCC(AG)AA(TC)CTCAT(GA)-AA(TC)CG
PD12 (anti-sense):	AGATTGTTGGTTAGCGTCAGC-TGA
PD18 (sense):	GAIAAG(AC)GTTT(C)TG(AC)-GGTTCCG
PD34 (anti-sense):	A(AG)(AG)AAIC(GT)(CT)TT(AG)-TC(TC)TG(AG)TAIGG
PD57 (sense):	TCAGA(GC)(GC)AG(GC)CI(CT)-TITA(TC)(AC)GIAA(AG)(AC)G
PD64 (sense):	ACTAGTCTGTGCCTCACCATC
PD75 (anti-sense):	TTCCC(AG)AA(CT)CTCAT(AG)-AAIC(GT)(CT)TT
PD90 (sense):	(CA)G(GA)CAGTT(TC)TA(TC)CGA-(TA)T(TC)GG(CT)(CA)G
PD92 (anti-sense):	CTCGGCTGTTTGGTTGGCACT

PD122 (sense):	TT(TC)ATG(CA)GTTT(TC)GGIAG-(AG)GGCGA
PD123 (anti-sense):	CTT(CG)CCGAACCTCATGAATCT-TTT
M13 rev (NEB # 1233):	AGCGGATAACAATTTACACAGGA
T3 promoter (NEB # 1228):	ATTAACCTCACTAAAGGGA

Peptide extraction

Ganglia were added to acetone to give a final concentration of 2 g/10 ml, and left at -20°C until processed further, but at least overnight. The acetone was decanted from the tissue, centrifuged, and the supernatant filtered through plain nylon (0.45- μm pore size; MSI, Westboro, Massachusetts). The clarified extract was reduced in volume to about 10% on a rotary evaporator with heating to 50°C , leaving a primarily aqueous phase that was again clarified by filtration.

HPLC fractionation

In all cases, the samples were pumped directly onto the reverse phase column (2.1×200 mm, Brownlee Aquapore Octyl) through the aqueous solvent pump. The absorbance of the effluent was monitored at two wavelengths (214 and 280 nm), and fractions (about 0.25 ml) were collected every 30 s. The flow rate was 0.5 ml/min throughout. Fractions to be rerun for further purification were pooled and diluted several-fold with the new aqueous solvent before being loaded onto the column. Three solvent systems were used: 0.1% trifluoroacetic acid (TFA) in water/0.1% TFA in 80% acetonitrile; the same with heptafluorobutyric acid (HFBA) substituted for TFA; and 5 mM sodium phosphate (pH 7.0) in water/5 mM sodium phosphate in 60% acetonitrile.

Radioimmunoassay

Radioimmunoassay, with either of two antisera (S253 or Q2), was used to analyze the fractions as previously described (Lesser and Greenberg, 1993).

Peptide synthesis

The peptide pQFYRFamide was custom synthesized by Research Genetics (Huntsville, Alabama). Other peptides were synthesized by the peptide synthesis laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research or purchased from Sigma Chemical Co.

Liquid chromatography-mass spectrometry (LC-MS)

Mass spectra were recorded in the positive ion mode on a TSQ-700 triple quadrupole instrument (Finnigan-MAT, San Jose, California) equipped with an electrospray ion source operating at atmospheric pressure. The

gctaacatcacaaagtcgacacaccacttgagtagagcgggactttgttagcacatttttagc	60
tcacgttttagtttctaagaaaaacgtctccacttcgccactcttcgatttggaaccag	120
catagcaccagggtgtacactcaagtctcacaagcaactacagatca	167
PD64	
ATG ACT AGT CTG TGC CTC ACC ATC GCC CCG GCC GTG CTC AGT	209
M T S L C L T I A P A V L S	
CTC ATC TGC CTG TCC TCG TAT GGG TGG GCT GAA GAC AAC AAC GGA ATC	257
L I C L S S Y G W A E D N N G I	
CAC ACA TTG GAC GAT GGA GAT AAT GAC CCA TTC TTC CGC CAC AAC AGG	305
H T L D D G D N D P F F R H N R	
CAG TTT TAC CGA TTC GGT AGA GCT TTC GTT CCT CTT TGG GAC AAT GCT	353
<u>Q F Y R F G</u> R A F V P L W D N A	
GAT GAC TCT TTA GTC CGG AAA AAC CTG CTG ACT CAT TGG TCT GAG TTT	401
D D S L V R K N L L T H W S E F	
CCC TTG TCA CCA GCT CTA GAT GAT GAT GTA TTT TCC AGA AAT AGC CGA	449
P L S P A L D D D V F S R N S R	
PD34	
CAG TTC TAC CGA TTC GGC CGC TCC TAC CCT <u>CCT TAC CAA</u> GAT AAA CGA	497
<u>Q F Y R F G</u> R S Y P P Y Q D K R	
TTT CTC CGG TTC GGA AGA TCT CAC CAG CCG GAT ATT GAT GAA TAT TTG	545
<u>F L R F G</u> R S H Q P D I D E Y L	
CAG GCC TTG AAT TCA GAC CAG GCT TAT AGG AAA AGG CGA TCA GAA	593
Q A L N S D Q A L Y R K R R S E	
GAT GGA GAT TCC AAA GAG GAT GGT CTG AAC CGA GTT GCC CGT <u>TCA GCT</u>	641
D G D S K E D G L N R V A R S A	
PD12	
<u>GAC GCT AAC CAA CAA TCT</u>	659
D A N Q Q S	

Figure 2. The nucleotide and deduced amino acid sequences of the 5' end of the FMRFamide precursor from *Helix aspersa*. The binding sites for the indicated primers (PD64, PD34, and PD12) are underlined. The portion of the sequence that is identical to the heptapeptide precursor is shaded, and the amino acids of the mature peptides (including the amide donor, glycine) are double underlined.

electrospray needle was operated at a voltage differential of 3–4 kV, and a sheath flow of 2 μ l/min of methoxyethanol was used. Scans were acquired every 3 s (Swiderek *et al.*, 1996).

The chromatography was performed on a microcapillary high-performance liquid chromatography (HPLC) system built at the Beckman Research Institute, City of Hope (Davis *et al.*, 1994, 1995). Fused silica columns with an inner diameter of 250 μ m were packed with Vydac 3- μ m C18 RP support. The sample was eluted with a gradient from 98% solvent A (0.11% TFA) to 62% solvent B (90% acetonitrile, 0.07% TFA) over 30 min with a flow of 2 μ l/min. The UV absorbance (200 nm) was monitored before the sample was introduced into the mass spectrometer. Spectra were generated by averaging scans containing the peak, and the masses were calculated with data reduction software (Finnigan MAT BIOMASS). The determined mass values of the molecular ions are accurate to within a few tenths of a dalton, reported values are rounded down to the nearest integral value.

Biological assays

The isolated heart. The perfused ventricle of *H. aspersa* was used as described previously (Lesser and Greenberg, 1993) to assay the cardioactivity of synthetic PQFYRFamide.

Recording from identified neurons. Electrical recordings were made from two identified neurons (C1 and C2) in the cerebral ganglia of *H. aspersa*; neither neuron is a known follower of a FMRFamide-containing cell (see Cottrell and Davies, 1987). The physiological solution had the following composition (mM): NaCl (90), KCl (5), MgCl₂ (5), CaCl₂ (7), HEPES (20), and the pH was adjusted to 7.4 with NaOH. Neurons were voltage clamped with a Dagan 8100 single electrode clamp system with low-resistance micropipettes containing 200 mM KCl. Peptide solutions (100 μ M) were prepared in an appropriate physiological medium and were usually pressure applied (Picospritzer II, General Valve Corporation). However, a barium-containing physiological solution (10 mM KCl, 25 mM BaCl₂, 5 mM MgCl₂, 75 mM tetraethylammonium chloride, 3 mM 4-amino pyridine, 5 mM Tris HCl, pH 7.5) was used during the measurement of currents passing through calcium channels, and the peptide was added to the bathing solution prior to the generation of a current-voltage curve.

Results

The complete sequence of the mRNA encoding the FMRFamide precursor

A partial sequence had previously been isolated from a *Helix aspersa* cDNA library; it extended from an

CGA CAG TTC TAC CGA TTC GGC	CGC TCC TAC CCT CCT TAC CAA GAT AAA CGA	H asp
AAG GG TG G T	A G C	H pom
	A G	C nem
T T A T	A A AGT C G A A T ATG GA	P acu
Arg Gln Phe Tyr Arg Phe Gly	Arg Ser Tyr Pro Pro Tyr Gln Asp Lys Arg	H asp
Lys Arg Leu	Ala	H pom
	Gln His Met Gly	C nem
Ile		P acu

TTT CTC CGG TTC GGA AGA TCT CAC CAG CCG GAT ATT GAT GAA TAT TTG ---	H asp
C T A	H pom
T A A T C C C TA A T A G C CT T GG CAT AAT	C nem
	P acu
Phe Leu Arg Phe Gly Arg Ser His Gln Pro Asp Ile Asp Glu Tyr Leu ---	H asp
	H pom
	C nem
Leu Asn Val Leu Asp Gly His Asn	P acu

--- --- CAG GCC TTG AAT TCA GAC CAG GCT TTG TAT	AGG AAA AGG CGA TCA	H asp
--- --- G T C	A	H pom
--- --- G T C	A G	C nem
ATT CCT TTT TTT GT C A GGC TT A C A C	G C A A G	P acu
--- --- Gln Ala Leu Asn Ser Asp Gln Ala Leu Tyr	Arg Lys Arg Arg Ser	H asp
--- --- Glu Ser His Pro		H pom
--- --- Glu Ser His		C nem
Ile Pro Phe Phe Val Gln Gly Val Lys Pro		P acu

GAA GAT GGA GAT TCC AAA GAG GAT GGT CTG AAC CGA GTT GCC CGT TCA GCT	H asp
G AT C G	H pom
G C A T C T A G C	C nem
TT A C A T CC AA C ACA CC AG TCA AT AA AG A A CAG C A	P acu
Glu Asp Gly Asp Ser Lys Glu Asp Gly Leu Asn Arg Val Ala Arg Ser Ala	H asp
Glu Tyr	H pom
Gly Thr Val	C nem
Leu Asn Ser Ala Asn Gln Thr Ala Glu Ser His Asn Glu Gln Pro	P acu

GAC GCT AAC CAA CAA TCT AAA AAT ACA CAA AGT AAC AAA TTT GGA AAG GAT	H asp
A T G A	H pom
T TT G TAC	C nem
ATA A TCC A G G G A A TCT A GAG G --- --- AAC AGC A A G	P acu
Asp Ala Asn Gln Gln Ser Lys Asn Thr Gln Ser Asn Lys Phe Gly Lys Asp	H asp
Thr Ile Glu Asn	H pom
Ser Leu Glu Tyr	C nem
Ile Asp Ser Lys Glu Thr Ser Lys Glu --- --- Asn Ser Lys	P acu

TTG CAA AAG AGG GAA ACA AAA AAG GAA AAG TTA AAT GCA AAT GAT GAT CTT	H asp
	H pom
	C nem
AT C A C A G C CCA CTT G A G T G AGC TC A --- ---	P acu
Leu Gln Lys Arg Glu Thr Lys Lys Glu Lys Leu Asn Ala Asn Asp Asp Leu	H asp
	H pom
	C nem
Tyr His Thr Pro Leu Glu Val Lys Ser Ile Asn --- ---	P acu

GAG ATT CTA TCA AAC GAG GAT --- GAT CTA GAA AAA AAG	H asp
C GCT AAG G G	H pom
T GT G AAG G G	C nem
A GGA ACT C T A A CAT C T T G G	P acu
Glu Ile Leu Ser Asn Glu Asp --- Asp Leu Glu Lys Lys	H asp
Thr Ala Lys Arg	H pom
Asp Val Val Lys Arg	C nem
Gly Thr Leu Lys His Phe Arg	P acu

TTT ATG AGG TTC GGA AAA CGT	H asp
C	H pom
C C C C A	C nem
	P acu
Phe Met Arg Met Gly Lys Arg	ALL

EcoRI site 21 nucleotide residues upstream of the tetrabasic sequence (RKRR; indicated on Fig. 1), through the 3' noncoding region (Lutz *et al.*, 1992). We have used the PCR-based strategy illustrated in Figure 1 to determine the sequence of the missing 5' end of the cDNA. This additional sequence (shown in Fig. 2), taken together with that of Lutz *et al.* (1992), completes the sequence of the FMRFamide precursor of *H. aspersa*. The precursor is diagrammed in Figure 1, and the complete sequence has been deposited in GenBank, Accession L20768.

The 5' end of the cDNA and the peptide precursor that it encodes [derived from these data and those of Lutz *et al.* (1992)] contain four noteworthy features (Fig. 1):

First, the 243 bases at the 5' terminal (including the noncoding region and the signal sequence) are identical to those of the cDNA encoding the precursor of the heptapeptide analogs of FMRFamide (Lutz *et al.*, 1992). Therefore, the two transcripts (*i.e.*, those encoding the FMRFamide and heptapeptide precursors, respectively) must be derived by alternative RNA splicing, a finding also reported for *Lymnaea stagnalis* (reviewed by Benjamin and Burke, 1994).

Second, downstream from the signal sequence, the 5' cDNA segment also encodes two copies of a previously unknown pentapeptide—pGlu-Phe-Tyr-Arg-Phe-NH₂ (pQFYRFamide)—as well as a copy of FLRFamide.

Third, 60 base pairs downstream from this FLRFamide, the cDNA encodes the tetrabasic cleavage site (Arg-Lys-Arg-Arg; RKRR) reported previously (Lutz *et al.*, 1992). The RKRR site also occurs in *Aplysia californica* (Taussig and Scheller, 1986) and *L. stagnalis* (Benjamin and Burke, 1994.)

Finally, 111 base pairs downstream, about $\frac{3}{5}$ of the distance between the tetrabasic (RKRR) site and the first copy of FMRFamide, the cDNA encodes a dibasic cleavage site (KR). This site is also found in *Aplysia californica* and *L. stagnalis* (Taussig and Scheller, 1986; Linacre *et al.*, 1990), but its relative position between the tetrabasic sequence and the first FMRFamide repeat varies.

In summary, the complete, derived precursor includes 10 copies of FMRFamide, 2 copies of FLRFamide (one in the midst of the FMRFamide repeats), 2 copies of the novel pentapeptide pQFYRFamide, and 2 conserved cleavage sites in addition to those flanking the FMRFamide-related peptides.

PCR amplification of other FMRFamide gene fragments

DNA from ganglia of the helioid snail *Cepaea nemoralis* and the camaenid snail *Polydonte acutangula* was prepared by crude alkaline lysis and amplified by PCR. In addition to the primers that had been employed for amplifications in *Helix*, we used another (PD92) that was synthesized to match exactly the *P. acutangula* sequence (see Fig. 1).

From *C. nemoralis* we obtained overlapping fragments covering much of the precursor, as shown in Figure 1 (for sequence see GenBank Accession U02488). The sequence of the *Cepaea* precursor is very similar to that of the two species of *Helix* (Figs. 1 and 3; Lutz *et al.*, 1992). Within the portion of the precursor that has now been sequenced from all three species, the *Cepaea* precursor has 88% nucleotide (nt) and 84% amino acid (aa) identity to *H. aspersa* and 90% nt and 81% aa identity to *H. pomatia*, and the two *Helix* species have 89% nt and 86% aa identity to each other.

The linear arrangement—in the precursors of *Cepaea* and *H. aspersa*—of the landmarks described above is virtually identical. In particular, the *Cepaea* precursor also encodes two copies of pQFYRFamide, a copy of FLRFamide, a tetrabasic site, and a dibasic cleavage site. Moreover, all of these features are in the same order and in virtually the same positions as in *H. aspersa*; the exception is that the distance between the dibasic cleavage site and the first copy of FMRFamide is one amino acid longer in *Cepaea* (Figs. 1 and 3). As expected, the segments between these landmarks are also very similar at both the amino acid and nucleotide levels (Fig. 3).

Although we have amplified only a small portion of the FMRFamide gene from *P. acutangula* (Fig. 1), most of the landmarks found in the cDNA from *H. aspersa* and *Cepaea* are present and recognizable. The 5' end of the sequence encodes a copy of FLRFamide, the tetrabasic site, and the dibasic cleavage site. Upstream from the FLRFamide is the site where PCR primer PD90 annealed; this primer was designed to hybridize to DNA sequences encoding either pQFYRFamide or pQFYRIamide. Although the PCR product that was cloned and sequenced contains the code for Ile, we cannot be sure which amino acid is actually coded for in the native DNA.

In contrast to the landmarks themselves, the segments

Figure 3. A detailed comparison of the nucleotide and amino acid sequences from the mid-portion of the FMRFamide precursors of *Helix aspersa* (H asp), *Helix pomatia* (H pom, taken from Lutz *et al.*, 1992), *Cepaea nemoralis* (C nem), and *Polydonte acutangula* (P acu). The nucleotide sequences are shown in italics, and the mature FMRFamide-related peptides are shown in bold. The sequences were aligned by the introduction of gaps (—) to keep the boxed common features (pQFYRFamide, tetrabasic site, Lys-Arg, and first copy of FMRFamide) in register. The complete nucleotide and amino acid sequences are shown only for *H. aspersa*. For the other species, only the differences from *H. aspersa* are indicated, except for the gaps; the gaps are represented by dashes wherever they occur.

between them are poorly conserved in *P. acutangula* (Fig. 3). The precursor in this species is only 41% identical to *H. aspersa* at the nucleotide level, and 34% at the amino acid level. Moreover, although the overall length of the region between the two outside landmarks—pQFYR (I/F) amide and FMRFamide (see Fig. 1)—is exactly the same as for *H. aspersa*, three off-setting gaps were required to align the tetrabasic and dibasic cleavage sites in the two species. For another example, the peptide just 5' of the tetrabasic site is especially dissimilar to that of *H. aspersa*: i.e., it is longer (23 amino acids instead of 20) and, even with the gaps, only 17% conserved.

Isolation of pQFYRFamide

The predicted peptide pQFYRFamide was synthesized; it was as reactive as FMRFamide in an RIA with antiserum S253 which facilitated the purification. The synthetic peptide eluted at 12–13 min in the TFA/ACN solvent system, i.e., coincident with a large peak of immunoreactivity observed with *H. aspersa* ganglion extracts (see Price *et al.*, 1990, for a typical pattern). Since this large peak also contains SDPFLRFamide and NDPFLRFamide, we sought a second solvent system that could separate pQFYRFamide from these two heptapeptides; the phosphate/ACN system proved to be satisfactory.

This fractionation procedure, with one modification, was applied separately to two *H. aspersa* extracts, one made from 50 pairs of cerebral ganglia and another from 30 subesophageal ganglia. The cerebral ganglion extract was given a preliminary fractionation with the HFBA/ACN system. Thereafter, each extract was fractionated on a TFA/ACN gradient, and the immunoreactive peaks from this run were further resolved with the phosphate/ACN solvent system. On this system, the cerebral ganglion extract showed a large peak at 19 min corresponding to synthetic pQFYRFamide, and a small peak at 16 min corresponding to the two heptapeptides (which are not separated in this system). The subesophageal ganglion extract contained peaks of heptapeptide (16 min) and pQFYRFamide (19 min) of roughly equal size (not shown). The pQFYRFamide peaks from both purifications were combined and run through a final TFA/ACN system. The major peak (12 to 12.5 min; Fig. 4A) was subjected to combined HPLC/mass spectrometry, revealing a molecular ion at 742 (Fig. 4B) in agreement with the calculated value of 742. Since this peptide eluted from the HPLC column at the same time as synthetic pQFYRFamide and had the same mass as pQFYRFamide, we conclude that it is pQFYRFamide.

Cardioactivity of pQFYRFamide

The effects of synthetic pQFYRFamide on the isolated heart of *H. aspersa* were compared with those of

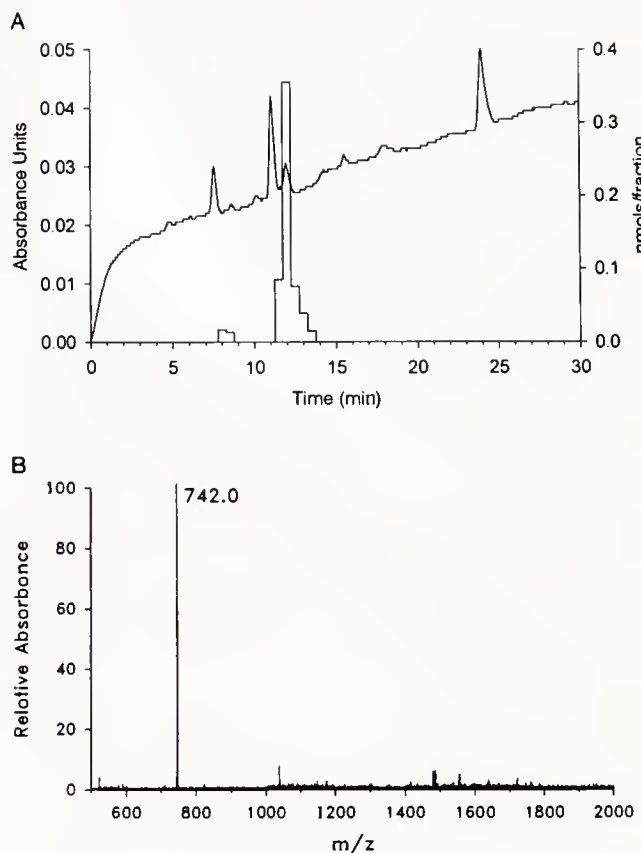


Figure 4. The purification and identification of pQFYRFamide. (A) The UV absorbance (214 nm) profile and a histogram of the immunoreactivity (determined with antiserum S253) in each fraction of the column effluent is shown for the final HPLC step (eluted with the TFA/ACN solvent system, see Methods). There is a delay of about 30 s between the UV recorder and the fraction collector for which no correction was made. (B) Mass spectral analysis of the main immunoreactive peak (12 min) from the fractionation illustrated in A. The observed m/z value (mass:charge ratio) of 742 corresponds to the calculated value for pQFYRFamide.

FMRFamide (Fig. 5). FYRFamide was about half as potent as FMRFamide, whereas pQFYRFamide was about 5 times more potent. Thus, blocking of the N-terminal by addition of pyroglutamic acid increases the potency of FYRFamide by 10-fold. Payza (1987) also noted that FMRFamide analogs with blocked N-terminals were more potent on the heart than those without.

Responses of identified neurons of *Helix aspersa*

Potassium current evoked in the C1 neuron. FMRFamide induces a slowly developing hyperpolarization of the C1 neuron that results from an increase in potassium conductance (Cottrell and Davies, 1987). In normal physiological solution (see Methods) pQFYRFamide evoked a response similar to that of FMRFamide ($n = 5$); e.g., under voltage clamp at -45 mV, an outward cur-

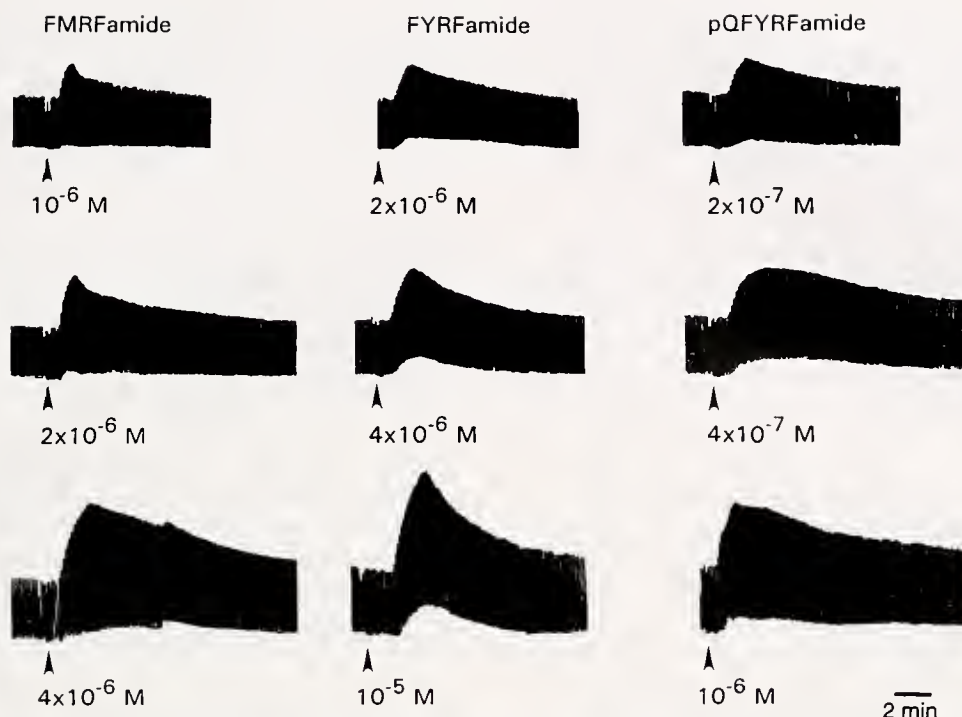


Figure 5. Representative recordings of the mechanical activity of an isolated perfused heart of *Helix aspersa*. A bolus (100 μ l) of the indicated concentration of peptide was introduced into the perfusing stream at the arrow; this volume corresponds to about 30 s of flow.

rent accompanied by an increased membrane conductance was recorded (Fig. 6A). At a holding potential of -100 mV the direction of the current response was inward (bottom record, Fig. 6A). The relationships between evoked current and holding potential were compared in normal physiological solution (containing 5 mM K) and in a saline with reduced potassium (1.5 mM), as shown in Figure 6B. The shift in reversal potential produced by reducing the potassium concentration was about 30 mV, close to that predicted for a response mediated entirely by a change in potassium conductance. FMRFamide and pQFYRFamide both had EC_{50} values of about 2 μ M, but pQFYRFamide consistently produced a larger maximum effect (about 50% greater than that of FMRFamide).

Suppression of a voltage-dependent calcium current in the C1 neuron. FMRFamide reduces the voltage-dependent calcium current by up to 30% (Colombaioni *et al.*, 1985; Cottrell and Lesser, 1987). pQFYRFamide also reversibly reduced the amplitude of the Ca current and by a similar amount ($n = 4$; data not shown). The EC_{50} for pQFYRFamide was similar to that for FMRFamide, i.e., about 5 μ M.

Fast sodium current evoked in the C2 neuron. FMRFamide was shown to produce a rapidly developing inward current in the C2 neuron (Cottrell and Davies,

1987), an effect mediated by the direct activation of sodium channels (Cottrell *et al.*, 1990; Green *et al.*, 1994). A comparison of the effects of FMRFamide and pQFYRFamide, recorded under voltage clamp from the whole cell (see Fig. 7), clearly shows that pQFYRFamide fails to evoke this fast inward current ($n = 7$). Apparently, pQFYRFamide cannot activate the FMRFamide-gated sodium channel.

Potassium current evoked in the C2 neuron. Some C2 neurons, but not the example shown in Figure 7, responded to FMRFamide with a weak outward current in addition to the fast response. This outward current, like the slow hyperpolarization observed in many *H. aspersa* neurons, is due to an increased potassium conductance (see C1 neuron above). pQFYRFamide also, and consistently ($n = 7$), evoked a slow outward current (Figs. 7 and 8A) that was accompanied by an increase in membrane conductance (Fig. 8A). This response inverted close to the potassium equilibrium potential (Fig. 8B) in both normal and reduced potassium solutions (based on $[K_i] = 98$ mM, Alvarez-Leefmans and Gamiño, 1982). It is, therefore, very similar to the slow increase in potassium conductance induced by FMRFamide and described above for the C1 neuron (Fig. 6; and Cottrell *et al.*, 1984); the EC_{50} values for pQFYRFamide and FMRFamide were similar—about 1 μ M.

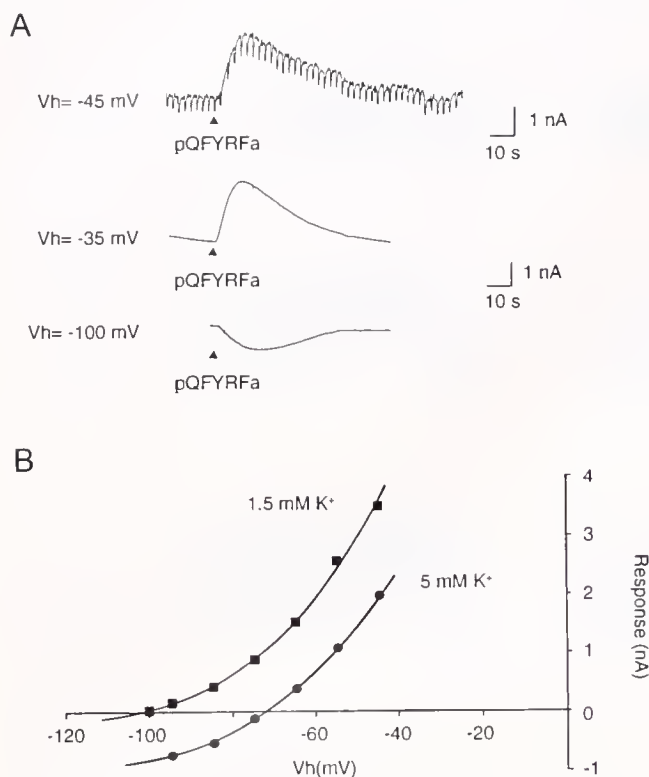


Figure 6. Effects of pQYRFamide on a C1 neuron from the cerebral ganglia of *Helix aspersa*. (A) Examples of the currents evoked by the peptide at different holding potentials (V_h). In the top record, the voltage was periodically stepped to -50 mV to monitor the conductance; note that the current pulses in response to the -5 mV steps are about twice as large at the peak of the response as in the control. (B) Relationship between peak evoked current and holding potential for responses in physiological solutions containing normal (5 mM, circles) or reduced (1.5 mM, squares) concentrations of potassium. The data shown in the graph were taken from an experiment on a single C1 neuron. A second experiment gave the same result.

Discussion

We have sequenced the 5' end of the cDNA encoding the FMRFamide precursor of *Helix aspersa*, and so the entire sequence is now complete. The general organization of this precursor is similar to that of the two other completely sequenced FMRFamide precursors: *Lymnaea stagnalis* (reviewed by Benjamin and Burke, 1994) and *Aplysia californica* (see Taussig and Scheller, 1986). The similarity is manifest in both the splicing pattern and in the linear arrangement of landmark sequences in the precursor.

The mRNA encoding the FMRFamide precursor in all three species is composed of at least two exons, and in all three the splice junction is in a roughly similar position. In both *Lymnaea* and *Helix* the first exon of the FMRFamide precursor is alternatively spliced to give another neuropeptide precursor (that of the FLRFamide-related heptapeptides), but no alternatively spliced product has ever been found in *Aplysia*.

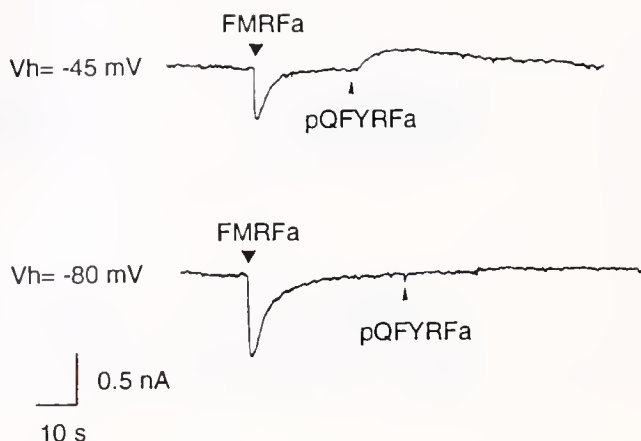


Figure 7. Comparison of currents evoked by FMRFamide and pQYRFamide in the C2 neuron. At a holding potential of -45 mV, pQYRFamide evoked an outward current, whereas FMRFamide evoked a fast inward current. Even at -80 mV, close to the reversal potential for the potassium response, no inward current in response to pQYRFamide was seen. Identical results were obtained in experiments in which the order of application was reversed (not shown).

The amino acid segments that are processed to become the individual mature peptides are not distributed at random in the precursors, rather, they are clustered according to their sequences in two domains. First, the C-terminal end of the precursor (always downstream of the tetrabasic sequence, RKRR) is the site of repeated segments, each comprising a FMRF sequence with its processing signals and acidic spacers. Second, the N-ter-

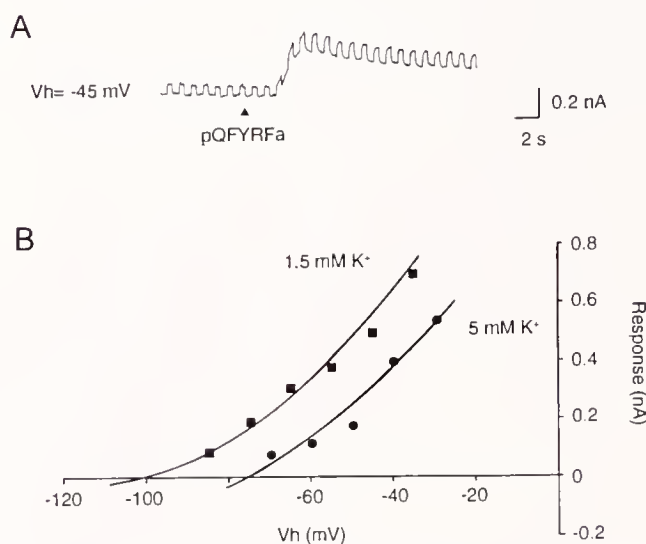


Figure 8. (A) The effect of pQYRFamide on the C2 neuron at -45 mV is accompanied by an increase in conductance as indicated by the increase in current pulses evoked by periodically stepping the voltage to -80 mV. (B) Relationship between peak current and holding potential (V_h) for responses evoked in physiological solutions containing either the normal (5 mM, circles) or reduced (1.5 mM, squares) level of potassium in an experiment on a single C2 neuron.

terminal domain (upstream of the tetrabasic sequence) is devoid of FMRFamide but always contains one or two copies of FLRFamide, as well as of related peptides. The related peptide in *H. aspersa* and *C. nemoralis* is the newly discovered pentapeptide pQFYRFamide (Fig. 1). In *Lymnaea*, one copy of pQFYRFamide is encoded at the level of the more 3' of the two copies of pQFYRFamide in *Helix*. And even in *Aplysia*, the 5' end of the FMRFamide mRNA encodes a copy of FLRFamide and a long, related peptide (Greenberg and Price, 1992).

The functional significance of the tetrabasic site and the sequences surrounding it has been considered from time to time (reviewed by Linacre *et al.*, 1990). In *Lymnaea*, a physiological role has been advanced for the acidic dodecapeptide segment SEQPD . . . SEEPLY that occurs just upstream from the tetrabasic site (Linacre *et al.*, 1990; Burke *et al.*, 1993). Our comparison of the *Polydonta* and *H. aspersa* precursors bears on this notion of function. Of the 23 amino acids preceding the tetrabasic site in these two land snails (and corresponding to the SEQPD . . . SEEPLY peptide of *Lymnaea*), only 5 are identical. Moreover, of the 23 residues following the tetrabasic site, only 3 are identical. These non-conserved sections of the precursor are, therefore, not likely to have a specific function common to all pulmonates, let alone all molluscs.

In contrast, the sequence of amino acids in the tetrabasic site seems to be conserved among pulmonates and opisthobranchs; moreover, it fits the consensus motif (Arg-Xaa-Lys/Arg-Arg) of a site of action for a furin-like endopeptidase (Hosaka *et al.*, 1991). The involvement of furin-like cleavage in neuropeptide processing was first demonstrated for the egg-laying hormone of *A. californica* (Newcomb *et al.*, 1988; Fisher *et al.*, 1988). Putative furin sites have since been observed in the myomodulin precursors of *Aplysia* (Miller *et al.*, 1993) and *L. stagnalis* (Kellett *et al.*, 1996); and the first cleavage seems to occur at such a site during the processing of the heptapeptide precursor in *H. aspersa* (Cottrell *et al.*, 1994). As for the tetrapeptide precursor, Linacre *et al.* (1990) had already suggested that the FLRFamide and FMRFamide domains could be separated by cleavage at the RKRR sequence. This conjecture was later elaborated by Greenberg and Price (1992), and it appears to be a reasonable working hypothesis.

If, however, this hypothesis is correct, and molluscan FMRFamide precursors are first processed into N- and C-terminal domains, then the products of the two domains might be functionally, and not merely structurally, distinct. In search of such a distinction, we compared the actions of pQFYRFamide (restricted to the N-terminal domain) with those of FMRFamide (restricted to the C-terminal domain) on the heart and selected neurons of *H. aspersa*.

The activity of pQFYRFamide on the isolated *Helix*

ventricle was qualitatively similar to that of FMRFamide and FLRFamide, the other two peptides derived from the tetrapeptide precursor. But pQFYRFamide, like FLRFamide, is about 5-fold more potent than FMRFamide (Price *et al.*, 1990).

On neurons, pQFYRFamide has some, but not all of the actions of FMRFamide. It mimics FMRFamide in evoking a potassium-dependent outward current and also in suppressing a voltage-activated calcium current. But pQFYRFamide—unlike FMRFamide—does not activate the fast inward sodium current in neuron C2, and thus it must not activate the FMRFamide-gated sodium channel. In fact, neither the heptapeptides (Cottrell and Davies, 1987), nor acetyl-Phe-Nle-Arg-Phe-NH₂, FYRFamide, or FLRF evoke this fast sodium current in the C2 neuron; and even FLRFamide is less potent than FMRFamide (K.A. Green, pers. comm.). Recently, the cDNA encoding this FMRFamide-gated sodium channel was sequenced and expressed in frog oocytes (Lingueglia *et al.*, 1995); these expressed channels had properties similar to those observed in the C2 neuron (Cottrell *et al.*, 1990; Green *et al.*, 1994).

The effectiveness of FMRFamide in evoking the inward sodium current—in contrast to the ineffectiveness of pQFYRFamide and FLRFamide—indicates that the recognition site for the fast ligand-gated response has a high level of specificity. More important, it shows that the pharmacological activity of the major peptide at the N-terminal portion of the precursor protein is different from the activity of the major peptides at the C-terminal portion, at least at one site.

In summary, we have sequenced the 5' end of the cDNA for the FMRFamide precursor of *H. aspersa*. When the FMRFamide precursors from different species were compared, the overall level of similarity varied with the phyletic distance between the species. But in every case, the sequences incorporating the FMRFamide-related peptides and the tetrabasic recognition site, as well as the organization of the precursor, were strongly conserved. Noting the putative peptide pQFYRFamide encoded on this cDNA segment, we searched for and subsequently identified this peptide in extracts of ganglia. Mass spectroscopy confirmed the predicted sequence and post-translational modifications (*i.e.*, amidation and cyclization of glutamine to pyroglutamic acid). pQFYRFamide had effects similar to those of FMRFamide on the heart and to an extent on neurons. But this novel pentapeptide did not activate the neuronal FMRFamide-gated sodium channel.

Acknowledgments

This study was supported by grants from NIH (HL28440 to MJG) and the SERC (to GAC). We thank Lynn Milstead for her help in preparing the figures.

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Evidence for Intercellular Coupling and Connexin-like Protein in the Luminescent Endoderm of *Renilla koellikeri* (Cnidaria, Anthozoa)

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Abstract. Gap junction plaques are abundant in Hydrozoa, where they play an important role in signal propagation through epithelia and nerve nets, but they have not been found in the two other classes of Cnidaria, the Scyphozoa and the Anthozoa. Here several lines of evidence are presented that point to the existence of intercellular coupling in tissues of the anthozoan *Renilla koellikeri*, especially in the luminescent endoderm. Dye-exchange experiments show that calcein vital stains spread between cultured cells after their reassociation. Polyp luminescence evoked by KCl depolarization, electrical stimulation, or β -adrenergic agonists was largely and reversibly suppressed in the presence of the gap junction uncouplers octanol, heptanol, and low pH sodium acetate. A connexin43-like protein was isolated on Western blots of *R. koellikeri* membrane extracts by using a monoclonal connexin-43 antibody. Loading this antibody in *R. koellikeri* tissues resulted in the suppression of luminescence evoked by electrical stimulation. Immunohistochemical investigations using this antibody revealed mostly punctate immunostaining associated with endodermal cells of the luminescent tissue and with the mesogleal nerve net. Electron microscopic observations confirmed the absence of conventional gap junction plaques in these tissues, but revealed the presence of tiny zones of close membrane apposition between light-emitting and other endodermal cells, with gaps of 2–4 nm. Taken together, these results are consistent with the notion of the existence in *R. koellikeri* of intercellular coupling (1) involved in local transmission of luminescence signals, and (2) mediated by connexin43-based

connexons that are not assembled into typical gap junction plaques.

Introduction

Gap junction channels are recognized as important pathways for intercellular communication subserving electrical and metabolic coupling in metazoan tissues (Paul, 1995). The wide range of roles attributed to gap junctions—maintenance of homeostasis, mediation of hormonal responses, and coordination of cellular activities in epithelial and neural conduction as well as in development—is reflected in the wide distribution of these channels among metazoan phyla (Revel, 1988). Within the phylum Cnidaria, however, gap junctions were reported to be abundant in the Hydrozoa, but absent in the Scyphozoa and the Anthozoa (Mackie *et al.*, 1984). Because cnidarians appear to be the lowest metazoans to possess gap junctions and nervous systems, this sharp dichotomy raises questions about the scope of intercellular communication in the classes Scyphozoa and Anthozoa.

In the Anthozoa, effector activities appear to be coordinated solely by simple nerve nets utilizing chemical synapses (Josephson, 1974; Satterlie and Spencer, 1987). One of these activities is bioluminescence in the colonial pennatulid *Renilla koellikeri* (Parker, 1920; Nicol, 1955; Anderson and Case, 1975), a behavior that probably serves as a deterrent to predators (Morin, 1983). Luminescence spreads over the colony as a wave that mirrors the spatio-temporal dynamics of excitation in the underlying colonial nerve net (Nicol, 1955; Anderson and Case, 1975). What is poorly understood, however, is how nerve-net activity translates into local luminescence ex-

Received 12 June 1996; accepted 4 September 1996.

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citation. A putative transmitter, epinephrine (E), specifically elicits luminescence in *R. koellikeri* (Anctil *et al.*, 1982) and has been detected in this species both by radioenzymatic methods and by high-performance liquid chromatography (HPLC) (De Waele *et al.*, 1987; Pani and Anctil, 1994a). Although the cellular distribution of E is still unknown, the presence of norepinephrine (NE)-immunoreactive nerve-net neurons (Pani *et al.*, 1995) and *in vivo* as well as *in vitro* evidence of enzymatic conversion of NE to E (Pani and Anctil, 1994b) support the existence of E-containing nerve-net neurons.

Several lines of evidence suggest that the adrenergic mediation of luminescence is indirect. First, E is ineffective in eliciting light emission directly from dissociated photocytes, the light-emitting endodermal cells (Germain and Anctil, 1988). Second, the characterized β 2-like adrenoceptors that have been associated with luminescence (Awad and Anctil, 1993) were localized by both autoradiography and *in situ* hybridization on "granular" cells adjacent to photocytes, but not on the photocytes themselves (Awad and Anctil, 1994). Lastly, although the endoderm of the muscular mesenteries of *R. koellikeri* is well innervated (Anderson, 1976), innervation of the circular musculature and of the luminescent endoderm is sparse (Satterlie *et al.*, 1976; Germain and Anctil, 1988). This raises the possibility that local transmission of nerve-net signals within the luminescent endoderm may involve pathways other than neural, possibly cell-to-cell coupling.

In another cnidarian luminescent effector where control appears to be indirect, that of the hydrozoan colony *Obelia*, photocytes depend on calcium entry through gap junctions from neighboring cells to trigger light emission (Dunlap *et al.*, 1987; Brehm *et al.*, 1989). Consideration of this alternative model of indirect control prompted us to examine the role of gap-junction-like coupling in luminescence regulation of the *Renilla* system and, more generally, to investigate the presence of specialized junctions and connexin-like proteins in the tissues of *R. koellikeri*. Having used multiple approaches to address this issue, we now report evidence that intercellular coupling involving a protein of the connexin family exists in *R. koellikeri*, and that this coupling plays a role in signal transmission throughout the luminescent endoderm.

Materials and Methods

Colonies of the sea pansy *Renilla koellikeri*, comprising polyps (autozooids and siphonozooids) rooted in a colonial tissue mass (rachis) prolonged by a peduncle, were obtained from Marinus Inc. (Long Beach, CA) and maintained in recirculated and aerated filtered seawater (Instant Ocean) at pH 7.6–8.0 and 13–15°C. The unfed animals were exposed to a photoperiodic cycle of 12 h: 12 h and used within 10 days of their arrival.

Effects of uncoupling agents on luminescence

Rachis pieces of about 5 × 5 mm (including autozoid polyps) were excised from colonies anesthetized in a 1:1 mixture of 0.37 M MgCl₂ and seawater. The excised tissues were maintained in artificial seawater (ASW: NaCl 395 mM, KCl 10 mM, CaCl₂ 10 mM, MgCl₂ 50 mM, and HEPES 10 mM, pH 8) until used. Luminescent responses were recorded by placing rachis pieces in a vial containing 1 ml of ASW, inserting the vial in the well of an LKB 1250 luminometer, and measuring the light output after injection of 0.5 ml of 0.53 M KCl in the vial with an LKB 1290 automatic dispenser. In some experiments, the β -adrenergic agonists isoproterenol and atenolol (both at 10 μ M) were substituted to the KCl solution. The luminescence signal (in millivolts) was digitized and stored for analysis with a BIOPAC MP100WS data acquisition system (Goleta, CA). Luminescent responses were quantified by integrating areas under the succession of flash curves recorded over a fixed period of time.

Three agents known to block gap junctions were used: 500 μ M heptanol or 250 μ M octanol in ASW, and sodium acetate seawater at pH 6.5 (Johnston *et al.*, 1980; Turin and Warner, 1980; Spray *et al.*, 1982). After one KCl-induced response was obtained in regular ASW, the polyps were transferred to any of the above solutions for 10 min and their response to KCl was again tested. The polyps were washed three times, 1 min per wash, in fresh ASW and subjected to another KCl challenge (recovery). The effect of these uncouplers on electrically induced luminescence was also tested. The same protocol was used except that trains of 10-ms pulses at 2 Hz delivered through a silver wire electrode in the luminometer vial were substituted for KCl depolarization.

To ensure that the uncoupling agents did not interfere directly with the luminescence effector of the photocytes, the latter were dissociated by incubating photocyte-rich autozoid tissues for 1 h in a medium containing 37.5 mg papain, 5 mg dithiothreitol (both from Sigma), 5 ml of ASW, and 5 ml of 0.37 M MgCl₂ at pH 8.0 (Holman and Anderson, 1991). After the suspensions were transferred into fresh ASW, the dissociated cells were subjected to KCl depolarization within 1–2 h of cell dissociation and their luminescent responses recorded as described above.

Dye-exchange experiments

Five sea pansies were anesthetized, their autozoid polyps were excised, and the polyp tissues were digested in the papain solution as described above. After the cell suspensions were transferred into fresh ASW, they were divided into two batches of equal volume per sea pansy, one of which was then loaded with 10 μ M calcein acy-

toxymethyl ester (calcein AM) and the other with 10 μ M calcein blue acetoxymethyl ester (calcein blue AM) (Molecular Probes, Eugene, OR) for 15 min at 12°C as prescribed by Leys (1995). These dyes were selected for their demonstrated ability to pass through gap junction channels (Tomasetto *et al.*, 1993) and for their propensity to become de-esterified inside cells, thus preventing their outward transmembrane diffusion. Cells were sedimented and resuspended in fresh ASW three times, then equal volumes of the two batches were mixed and the cells left to reassociate overnight at 12°C in plastic petri dishes. Just before reassociation, some batches of mixed cells were divided into two equal volumes, sedimented again, and resuspended either in fresh ASW (controls) or in ASW with 250 μ M octanol. Aliquots (0.1 ml) of cell-aggregate suspensions were transferred onto microscope slides, coverslipped, and examined with a Wild-Leitz DIALUX 20 fluorescence microscope. Incidence of dye exchange was assessed by scoring aggregates of three or more cells displaying at least one cell with dual calcein AM (green) and calcein blue AM (blue) fluorescence within a 1 cm² area.

Western blot detection of connexin43-like protein

Sea pansy colonies were anesthetized as described above and autozooid polyps, as well as rat heart tissues, were finely minced. Tissues were suspended in 15 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and homogenized with a Polytron on ice. They were centrifuged at 100 $\times$ g for 5 min to discard large debris and, after the supernatant was filtered through cheesecloth, recentrifuged at 35000 $\times$ g for 20 min at 4°C. The supernatant was discarded and the pellets were resuspended in the Tris-HCl buffer without sucrose and recentrifuged at 35,000 $\times$ g and 4°C for 15 min. This latter procedure was repeated three times. The resulting pellets were transferred to SDS reducing sample buffer (0.125 M Tris buffer with 5% SDS, 10% mercaptoethanol, 20% glycerol, and 0.005% bromophenol blue) and run in 12% SDS-polyacrylamide gels with a Bio-Rad Mini-Protein II cell according to Laemmli's (1970) method. The gels were stained with 2.5% Coomassie blue in 50% methanol and 7% acetic acid.

Immunoblots were obtained by electrophoretic transfer of gels to PUDF membranes (Bio-Rad) with 0.2- μ m porosity. After exposing the blots to 3% bovine serum albumin in Tris-buffered saline (TBS; 20 mM Tris HCl with 0.5 M NaCl, pH 7.2) for 4 h to block unspecific antibody binding, they were incubated at 4°C for 24 h in a 1:1000 solution of a monoclonal anti-connexin43 (anti-Cx43) antibody (Chemicon International) in TBS. This antibody was raised against a synthetic peptide corresponding to aminoacid positions 252–270 of rat heart

Cx43. That fragment is part of the COOH terminus of the protein on the cytoplasmic side, which diverges considerably among connexins (Beyer *et al.*, 1987). Blots were washed three times, 15 min per wash, in TBS and reacted with a Vectastain goat anti-mouse ABC peroxidase kit (Vector). Some blots were stained with Coomassie blue for direct comparison of protein profiles.

Effect of Cx43 antibody on luminescence

Small pieces of colonial tissue (rachis) bearing one autozooid polyp and several siphonozooid polyps (also containing photocytes) were excised from unanesthetized colonies and placed individually in assay vials containing 150 μ l of ASW. To stimulate these preparations, the denuded tip of a fine, Teflon-coated silver wire was inserted in the rachis piece and another, immersed in the surrounding ASW, served as the reference electrode. The assay vial was inserted in the lightproof well of a Berthold Biolumat LB 9500T luminometer, and the opposite tips of the electrodes were connected to a Grass S9 stimulator. Spontaneous luminescent activity was first recorded as integrated light output (in millivolts) over a 30-s period. The luminescent response to a train of 10 supra-threshold monopolar pulses (25–40 V, 10 ms each, 2 Hz) was then recorded, again as integrated light output over the first 30 s after the initial stimulation pulse. The background signal of the luminometer was automatically subtracted from the measured luminescent activity.

After control responses were recorded, the ASW was removed from the assay vial and substituted with 150 μ l of an ice-cold solution containing the monoclonal Cx43 antibody diluted 1:20 in ASW with 5% dimethylsulfoxide (DMSO) to allow the antibody to penetrate cells (Fraser *et al.*, 1987). Incubation was run at room temperature. Luminescent responses to electrical stimulation were recorded as above, 30–45 min after onset of incubation, then at later times specified in the Results to monitor the recovery of responses as the antibody presumably deteriorated in the tissues.

Data were analyzed by computing the ratio of stimulated over unstimulated (spontaneous) luminescent activity for each experimental condition (control and various times after exposure to antibody). This normalization was made necessary by the great variability of absolute light output between preparations. To compare ratios obtained from controls with those from antibody exposures in the same preparation, paired *t* tests were performed when necessary and significance level was set at *P* < 0.05.

To rule out nonspecific effects due to membrane permeabilization, unstable electrode insertion, or fatigue of preparation, we undertook sham experiments by submitting preparations to treatments as above except for

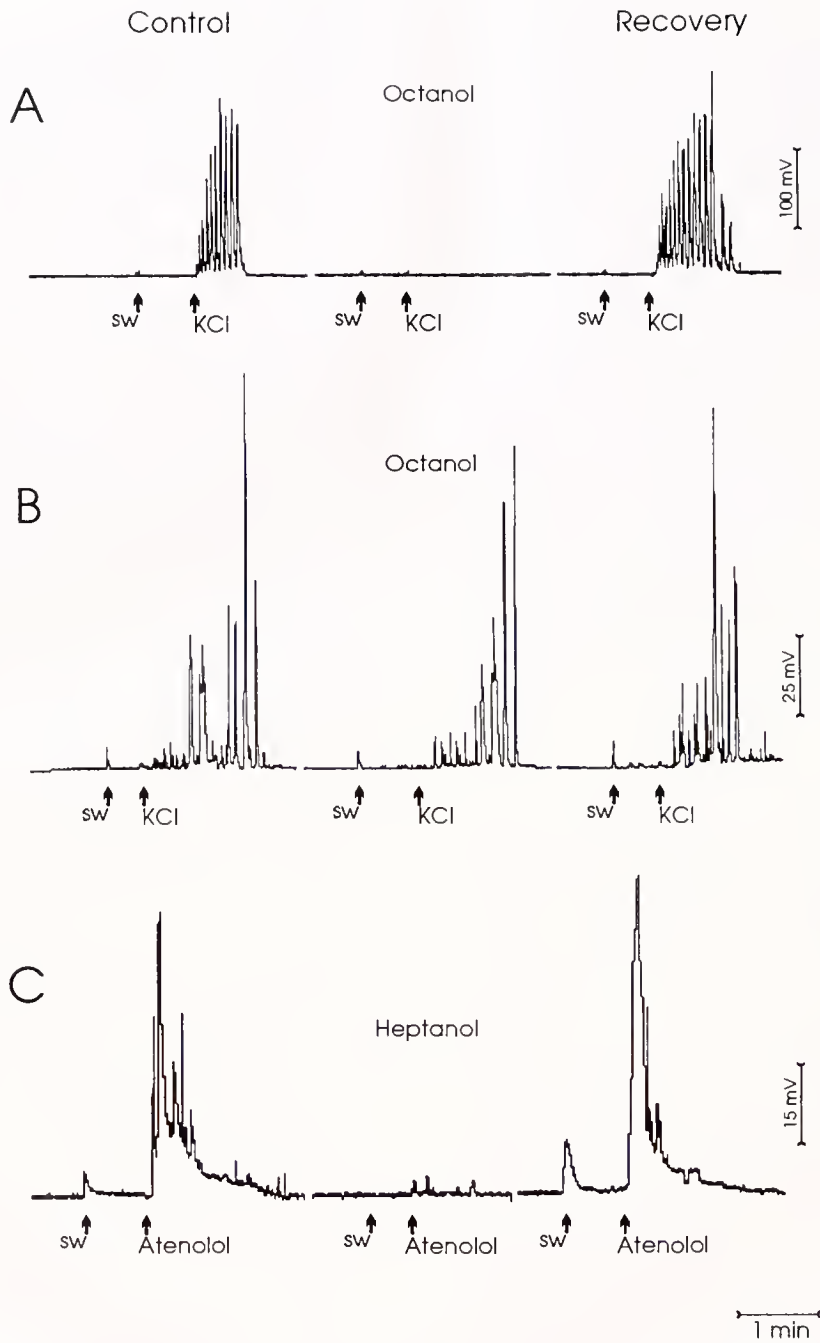


Figure 1. Photometric recordings of the effect of gap junction uncouplers on stimulated bioluminescence of *Renilla koelikeri*. (A) Effect of 0.25 mM octanol on *in situ* KCl-induced flash activity. (B) Effect of 0.25 mM octanol on KCl-induced flash activity of dissociated photocytes. (C) Effect of 0.5 mM heptanol on *in situ* luminescence induced by 10 μ M atenolol. Spurious mechanical stimulation induced by delivery of 0.5 ml seawater (sw) was monitored throughout the experiments.

substituting fresh ASW with 5% DMSO, instead of the antibody solution, for the regular ASW of control conditions. To rule out spurious effects of macromolecules inside cells, we substituted a goat antimouse immunoglobulin (Jackson Immunochemicals), diluted 1:20 in ASW

with 5% DMSO, for the Cx43 antibody in the assay vial. To verify that the effect was possible only through an intracellular route, the preparation was exposed to a Cx43 antibody solution lacking DMSO. To directly demonstrate antibody penetration, three preparations loaded

with connexin43 antibody were fixed, processed for freeze sectioning, and the sections reacted with the secondary antibody as described below.

Localization of Cx43 immunoreactivity

Autozooid polyps were excised from anesthetized colonies as above, and immersed in freshly prepared Zamboni's fixative (4% paraformaldehyde and 7.5% saturated picric acid in phosphate buffer with 2.4% NaCl, pH 7.4) for 24 h at 4°C. After three rinses in PBS, followed by immersions in PBS with 15% and 30% sucrose, the polyps were embedded in O.C.T. compound (Miles) and frozen by immersion in dry-ice-chilled isopentane. Sections 15 μ m thick were made with a Hacker-Bright cryotome. The sections were rinsed in three 15-min changes of PBS, followed by pretreatment in PBS with 0.2% Triton-X-100 and 1% bovine serum albumin (BSA). Incubation was carried out for 24 h at 4°C in the Cx43 monoclonal antibody diluted 1:200 or 1:100 in PBS with Triton-X-100 and BSA as above. After rinsing in PBS as above, sections were incubated in PBS for 1 h at room temperature in Cy3-conjugated goat anti-mouse immunoglobulin diluted 1:100. Sections were then rinsed in PBS as above and mounted in a 1:3 mixture of glycerol-PBS before being examined with a Wild-Leitz Dialux 20 fluorescence microscope equipped with a rhodamine filter. Control sections were processed as above except for omitting the primary antibody from the incubation solution.

Electron microscopy

Autozooid polyps were excised from anesthetized colonies as described above. They were immersed in a freshly prepared fixative solution containing 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) with 2.4% NaCl for 1 h at room temperature. After rinsing tissues in buffer, they were postfixed in 1% OsO₄ in cacodylate buffer for 1 h. Tissues were dehydrated in graded ethanol and propylene oxide, and embedded in Epon 812. Sections were cut with a diamond knife on an LKB ultramicrotome and deposited on 100- and 200-mesh stainless steel grids (JBEM Inc.). The sections were counterstained with uranyl acetate and lead citrate and examined with a JEOL-100S electron microscope.

Results

Effect of gap junction uncouplers on evoked luminescence

The light emission evoked in sea pansy autozooids by KCl depolarization appeared as a series of flashes of varying amplitude and frequency (Fig. 1A,B). These re-

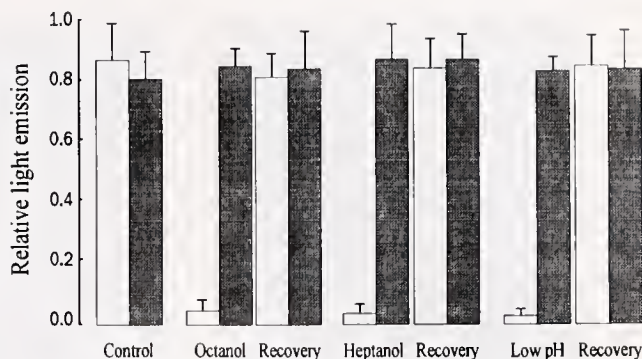


Figure 2. Histogram summarizing data on the effect of exposure to gap junction uncouplers on KCl-induced bioluminescence of intact tissues (open bars) and dissociated photocytes (shaded bars) of *Renilla koellikeri*. Data are expressed as means $\pm$ SE ($n = 6$ experiments) of light output ratio relative to controls.

sponses were either considerably reduced or abolished in a reversible manner in the presence of octanol, heptanol, or sodium acetate-seawater (Figs. 1A, 2). Photocyte-enriched dissociated cell preparations responded to KCl depolarization with series of irregular flashes (see also Germain and Anctil, 1988). In contrast to responses of photocytes *in situ*, the KCl response of dissociated photocytes was unaffected in the presence of octanol or heptanol (Figs. 1B, 2). Field electrical stimulation induced facilitated flashes on a one-to-one basis with stimulation pulses, and this type of response was also largely and reversibly abolished in the presence of any of the three uncouplers (not shown). Stimulation of the β 2-like adrenergic receptors of the sea pansy is known to induce bioluminescence (Awad and Anctil, 1993). Exposure of tissues to isoproterenol, a nonselective β -adrenergic agonist, or to atenolol, a selective β 2-adrenergic agonist in the sea pansy (Awad and Anctil, 1993), induced luminescence in the form of glows and superimposed flashes, a response that was greatly and reversibly reduced in the presence of heptanol (Fig. 1C).

Dye exchange

Cells loaded with calcein AM or calcein blue AM were strongly fluorescent, and their fluorescence did not fade significantly over a period of 24 h. When cells loaded with either of the two dyes were allowed to reaggregate for 15 h, many of them exhibited both the green fluorescence of calcein AM and the blue fluorescence of calcein blue AM (Fig. 3). Among those were identifiable cells such as endodermal granular cells known to harbor luminescence-associated β 2-like adrenergic receptors (Awad and Anctil, 1994) and nerve cells (Fig. 3). Of 1465 cell clusters (three cells or more) scored in 10 aliquots of mixed cell suspensions from three sea pansy colonies,

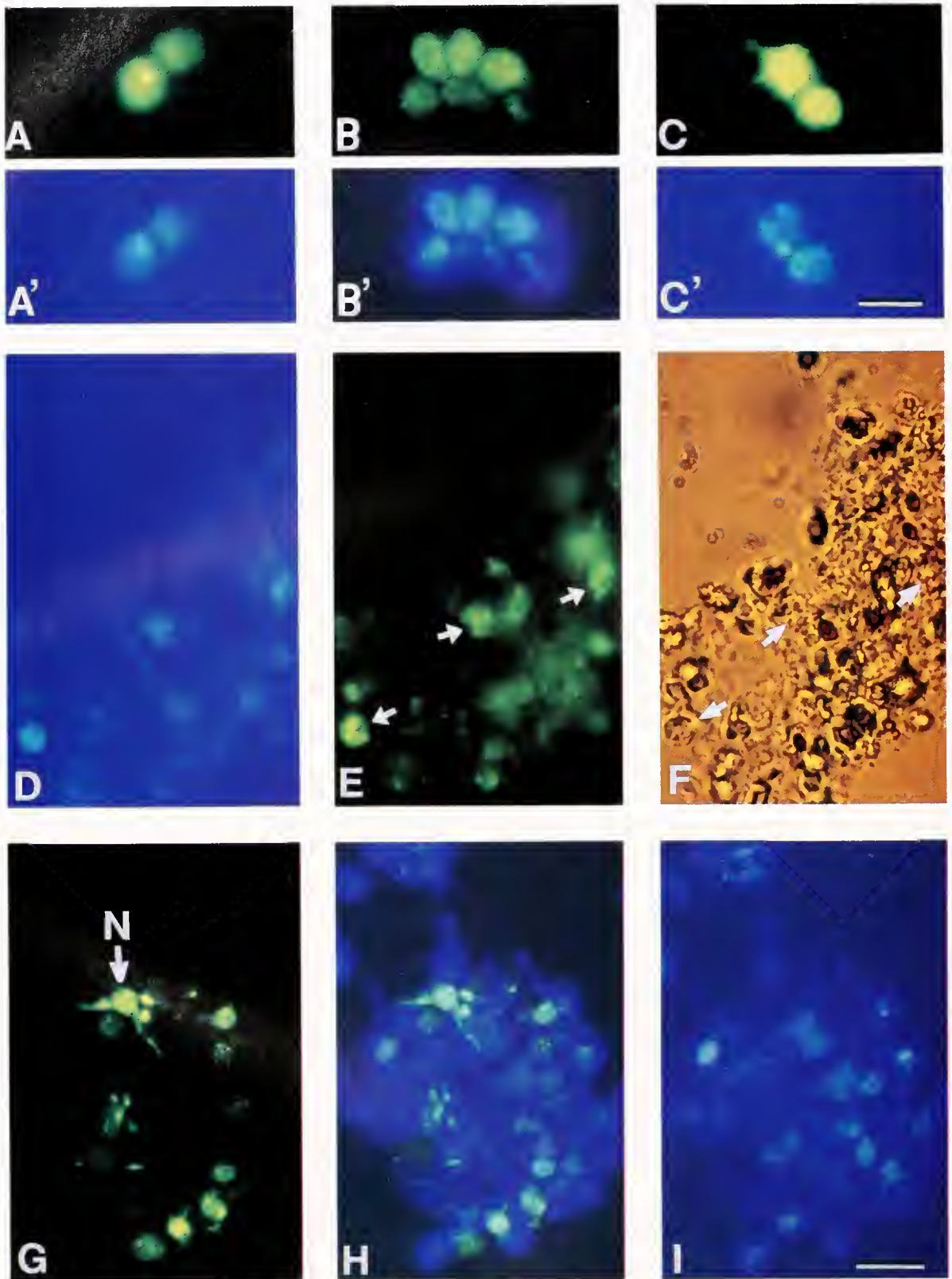


Figure 3. Demonstration of dye transfer between reaggregated sea pansy cells. Dissociated autozooid polyp cells were loaded separately with calcein AM and calcein blue AM and plated together to allow reaggregation and dye passage between cells within a reaggregate. Upper panel shows dye exchange between

two-thirds [$66.5\% \pm 3.9\%$ ($X \pm \text{SEM}$, $n = 10$ aliquots)] included at least one cell in which the dyes were co-localized. In contrast, mixed cell suspensions allowed to reaggregate in the presence of $250 \mu\text{M}$ octanol for 15 h showed zero incidence of dye exchange. In addition, the incidence of cell clusters was reduced to one-third ($30.7\% \pm 4.9\%$, $n = 5$ aliquots) of that in untreated suspensions from the same cell batches. A partial reversal of the octanol effect was recorded when reaggregation was prolonged to 48 h, with low incidence of dye exchange ($13.9\% \pm 2.2\%$, $n = 5$) and an increase in the treated/untreated ratio of cell aggregates ($44.2\% \pm 5.8\%$, $n = 5$).

Detection of a connexin-like protein

Because the results from experiments with uncouplers and dye exchange suggested the involvement of gap-junction-like communication in the sea pansy, we next looked for gap junction proteins. We chose to focus on Cx43 because preliminary Northern blot analyses in another anthozoan, the sea anemone *Haliplanella*, had revealed hybridization products with rat heart Cx43 but not with rat liver Cx32 cDNA (Hessinger *et al.*, 1992).

As shown in Figure 4 (lanes 1 and 2), Western blots of membrane protein extracts from the sea pansy revealed two antigen bands that bound the Cx43 antibody, one with an apparent molecular weight of ~ 43 kDa and another with twice that molecular weight. Both bands co-electroeluted with the rat heart 43 kD connexin and its presumptive dimer (Fig. 4, lane 3). The rat heart bands had a density more-or-less similar to those of the sea pansy.

Effect of Cx43 antibody on luminescence

The antibody was successfully and consistently introduced into cells of the sea pansy by permeabilization with DMSO; this result was demonstrated by visualization of a diffuse tissue fluorescence in loaded preparations that were fixed after experiments and reacted with Cy3-conjugated goat anti-mouse antibody. Under our loading conditions, the tissues responded normally to electrical stimulation with facilitating flashes as reported above. Whether in the presence or absence of 5% DMSO,

the unloaded preparations also exhibited a facilitation of the integrated luminescent response to a second challenge 30–45 min after the first (Fig. 5).

The luminescent responses of all preparations loaded for 30 min with the Cx43 antibody were nearly abolished for at least 90 min (Fig. 5). There was a partial but significant recovery 5–7 h after loading, presumably due to antibody degradation, and another response decline more than 10 h later, probably resulting from tissue deterioration (Fig. 5) brought about by conducting the experiments at room temperature. Loading preparations with a goat anti-mouse immunoglobulin instead of the Cx43 antibody or exposing preparations to the Cx43 antibody without DMSO did not significantly alter the luminescent response. Moreover, exposing tissues to the antibody solution did not, by itself, elicit any luminescence.

Immunohistochemical localization of Cx43-like antigen

Because it seemed that the Cx43 antibody specifically recognized an antigen behaving like rat heart Cx43 in Western blots and acted like a gap junction uncoupler to block luminescent responses, we undertook to determine by immunohistochemistry whether Cx43-like immunoreactivity is present in the photocyte-rich endoderm.

Specific Cx43 immunoreactivity was widespread but more abundant in the endoderm (Fig. 6A–C) and in the mesogleal nerve-net (Fig. 6D) than in the ectoderm. Immunofluorescence was lost in sections unexposed to the primary antibody; the only exceptions were some ectodermal gland cells that exhibited nonspecific reactivity. In both autozooid and siphonozooid polyps, photocytes are clustered in specific locations of the endodermal layer where cells filled with highly refringent granules (presumably acting as diffuse reflectors for the light emission) predominate (Anctil *et al.*, 1984; Awad and Anctil, 1994). In autozooids these locations are at the base of each of the eight tentacles and at the base of the corresponding eight septa of the autozooid column (Morin, 1974). It was in these parts of the endoderm that the density and intensity of immunoreactive sites appeared to be the greatest (Fig. 6A,B).

The other site of abundant immunoreactivity was the

all cells of three small clusters, with view of calcein AM dye (A–C) and calcein blue AM dye (A'–C'). Cells in C,C' are granular endodermal cells. Middle panel shows a large cluster of cells in which only a few cells exchanged dye, such as the three cells filled with calcein blue AM (D) that are indicated by arrows in views of their calcein AM fill (E) and of the cell mass in bright field (F). Note in F the presence of symbiotic brown unicellular algae. Lower panel shows a cell cluster from a preparation in which cells were allowed to reaggregate in the presence of $250 \mu\text{M}$ octanol. G and I are views of calcein AM and calcein blue AM dyes, respectively, and H is a double exposure of both dyes. N is a neuron with cilium and bifurcating axon. Note that no dye exchange occurred. Bar in upper panel = $15 \mu\text{m}$, and bar in lower panel = $20 \mu\text{m}$ (D–I).

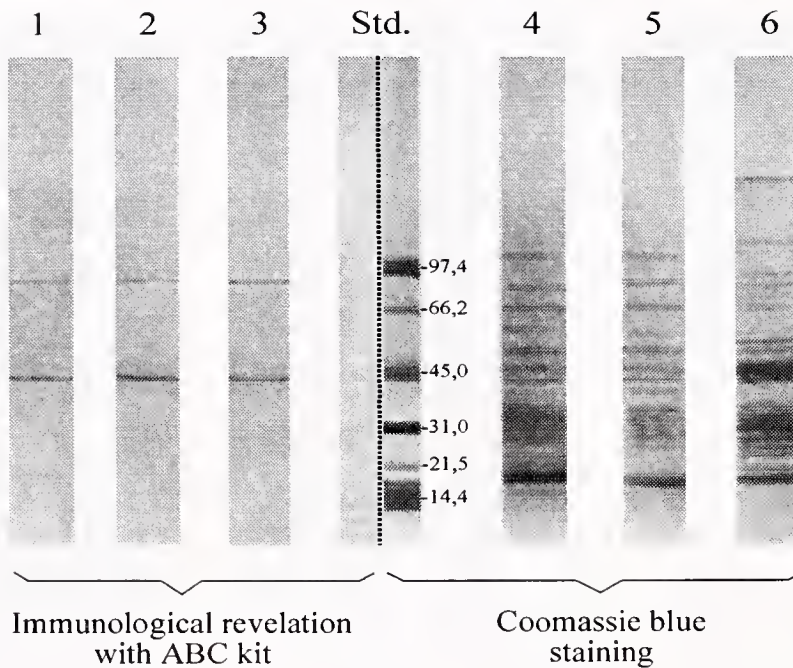


Figure 4. Immunoblot analysis of connexin43-like protein in sea pansy membrane homogenates. Lanes 1 and 2 show binding of monoclonal Cx43 antibody to the 43 kDa monomer and the 86 kDa dimer from homogenates of two sea pansies, and lane 3 shows the same from a rat heart homogenate. Lanes 4–6 are Coomassie blue stained blots from the homogenates of lanes 1–3, presented in the same order.

mesogleal nerve net, a meshwork of neurons and amoebocytes embedded in the collagen-based jelly, the mesoglea, that separates ectoderm from endoderm (Fig. 6D). Unlike the luminescent endoderm, in the mesoglea immunoreactivity was inconsistent, being strong in some locations (Fig. 6D), weak or absent in others. The immunoreactivity appeared as tiny fluorescent dots distributed in stringlike fashion along tracts of amoebocytes and neurites. The size of the fluorescent dots was in the sub-micrometer range. Lack of resolution made it impossible to determine if the immunoreactivity was associated with either amoebocytes or nerve cells, or both.

The pattern of immunoreactivity in the luminescent endoderm was polymorphic. Reactivity usually appeared as punctate immunofluorescence (Fig. 6A), as expected of minute structures the size of gap junctions and as observed in tissues examined in this way, including those of the cnidarian *Hydra* (Fraser *et al.*, 1987). The fluorescent dots varied in diameter but, as in the mesoglea, all were in the submicrometer range. Owing to the thickness of the sections and the multifocal planes where these small cells are located, the distribution of the reactive dots appeared generally random. Occasionally, however, cells containing refringent granules appeared to be associated with punctate immunoreactivity at their boundaries (Fig. 6A). Less frequently, immunoreactive endodermal cells were

individually recognizable, perhaps because contiguous cells lacked reactivity and allowed an unencumbered view. In this case the immunofluorescence was associated with cell boundaries, cell contour fluorescence appearing as a fine threadlike assembly (Fig. 6C).

Ultrastructural observations

The photocyte-rich endoderm was examined by electron microscopy for evidence of specialized contacts between cells. An exhaustive search failed to uncover any structure corresponding to descriptions of conventional gap junctions such as those described in hydrozoans (Hand and Gobel, 1972; King and Spencer, 1979). However, in addition to septate junctions (Fig. 7A), small zones of close membrane apposition were frequently seen between endodermal cells (Fig. 7A–C). These contact zones were observed between a variety of endodermal cells, including granular cells (not shown), myoepithelial cells (Fig. 7A), photocytes (Fig. 7B,C) and digestive cells (Fig. 7C). These generally had a contact area of 20–50 nm and an intermembrane gap of 2–4 nm, but lacked any other specialization except for densities in the gap zone. Less frequently, larger contact zones, up to 100 nm in length with intercellular gap spaces of 5–6 nm, were characterized by gap densities of regular periodicity (Fig. 7B).

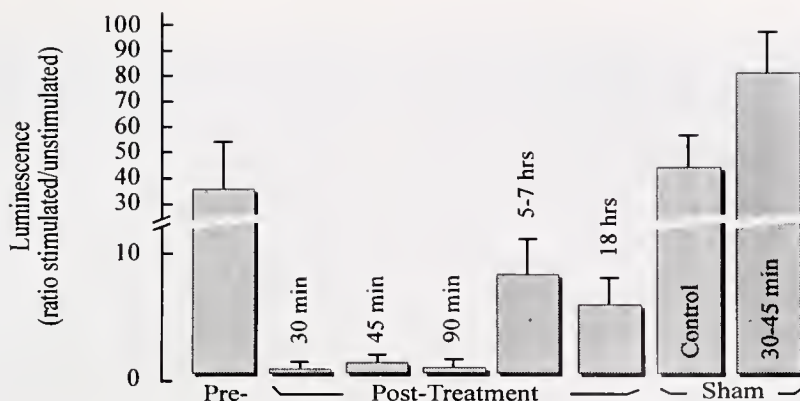


Figure 5. Effect of Cx43 antibody treatment on bioluminescence elicited in sea pansy tissue preparations by electrical stimulation. Pretreatment represents response to electrical stimulation in normal ASW immediately before onset of antibody penetration experiments. Sham represents experiments in which pretreatment was as before (control) but treatment consisted of exposing the preparations to ASW + 5% DMSO. Data from responses 30 and 45 min after exposure were pooled. Bars represent means $\pm$ SEM ($n = 6$, except at 5–7 h and 18 h when $n = 4$ and 3, respectively).

Discussion

The results of this study provide converging evidence for the existence of a connexin-like gap junction protein in the sea pansy and for the involvement of connexon-mediated intercellular coupling in the transmission of luminescence effector signals. This and other evidence (Hessinger *et al.*, 1992), while confirming the failure to observe typical gap junction plaques in anthozoans, challenges the notion that anthozoan cells “have little if any capability for direct electrical or metabolic communication” (Mackie *et al.*, 1984).

Intercellular coupling occurs and is necessary for mediation of luminescence signaling

The conventional demonstration of dye coupling after intracellular injection of Lucifer yellow was not feasible because of the technically challenging small size and instability of penetrated sea pansy cells. Our prior experience with suspensions of dissociated sea pansy cells indicated that reassociation into clusters of varying sizes occurred. We took advantage of this property in experiments designed to allow exchange of two vital dyes (calceins) between reassociated cells. The observation that a substantial number of cell clusters included cells doubly labeled with calcein AM and calcein blue AM under these conditions indicates that dye exchange occurred and, consequently, that some form of coupling is effected between these cells. The possibility that dye colocalization results from dye leaking out of cells and into cells loaded with the other dye is highly unlikely. The calcein dyes are noteworthy for their effective retention inside viable cells and no evidence of leaching, such as reduc-

tion of the fluorescent signal, was observed in the course of these experiments. The suppression of dye exchange in the presence of the uncoupler octanol also reinforces the notion that dye exchange occurred via connexon-related channels. It should be emphasized, however, that these results provide strong evidence that coupling is feasible between reassociated sea pansy cells; whether this coupling in fact occurs in intact tissue remains to be resolved.

Luminescent responses of intact autozooids to KCl depolarization, β -adrenergic activation, and electrical stimulation were all inhibited in a reversible manner by octanol, heptanol, and low pH (6.5). These agents are known for their blocking effect on coupling *via* gap junctions (Johnston *et al.*, 1980; Turin and Warner, 1980; Spray *et al.*, 1982) and have perturbing effects on the ultrastructure of connexon arrangements (Délèze and Hervé, 1983). They not only inhibited the KCl-induced luminescent response of endodermal cell clusters of *Obelia*, but also blocked the intercellular spread of injected Lucifer yellow in these preparations (Dunlap *et al.*, 1987). The possibility exists that the observed effects are due to interference with participants in the luminescent system other than intercellular coupling. This is unlikely, however, because the luminescent responses of dissociated photocytes were unaffected by the uncoupling agents. If KCl depolarized photocytes directly in intact tissues, uncoupling agents would not be expected to affect luminescence. The effectiveness of uncoupling agents on *in situ* luminescence suggests that KCl triggers luminescence only *via* nerve-net depolarization as occurs following electrical stimulation. If KCl directly depolarized photocytes, a sustained glow response would

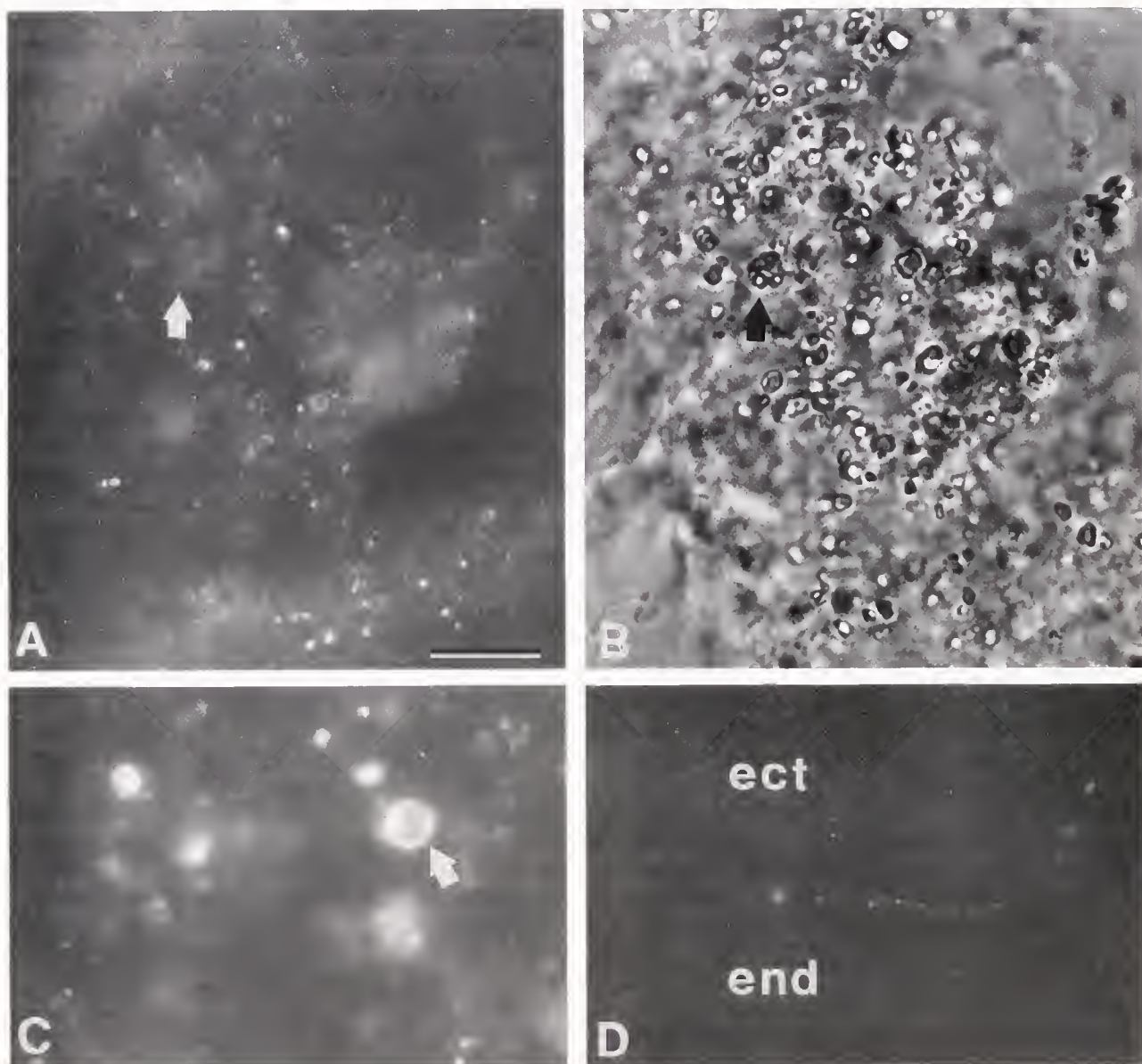


Figure 6. Connexin43 immunofluorescence in the luminescent endoderm (A–C) and mesoglea (D) of autozooid polyps of the sea pansy. (A) Punctate connexin43 immunofluorescence in luminescent endoderm. (B) Phase contrast view of field in A, showing details of refringent granule distribution and morphology. Note that granular cell by arrow in B lacks immunofluorescence in A (arrow) except for apparent punctate reactivity along its contour. (C) Endodermal cells with immunofluorescent contour, one of which (arrow) is in sharp focus and exhibits a string of tiny dots. (D) String of punctate immunofluorescence in mesogleal amoebocyte tract (in which lies the nerve net), sandwiched between non-immunoreactive ectodermal (ect) and endodermal (end) layers. Calibration bar = 10 μ m (A–C), 9 μ m (D).

be expected; instead, KCl produced phasic luminescent responses. Thus the necessity of the integral luminescent tissue for the blocking effect of the uncouplers and the consistency of action of all three uncouplers on luminescent responses obtained by different means can best be explained by the involvement of some form of intercellular coupling in mediating the luminescent signal.

A connexin43-like protein is present in sea pansy tissues

If intercellular coupling sensitive to gap junction blockers is involved in sea pansy luminescence signaling, then one should be able to detect a protein capable of assembling into connexons for this coupling. There is *a priori* no reason to assume that such a protein should

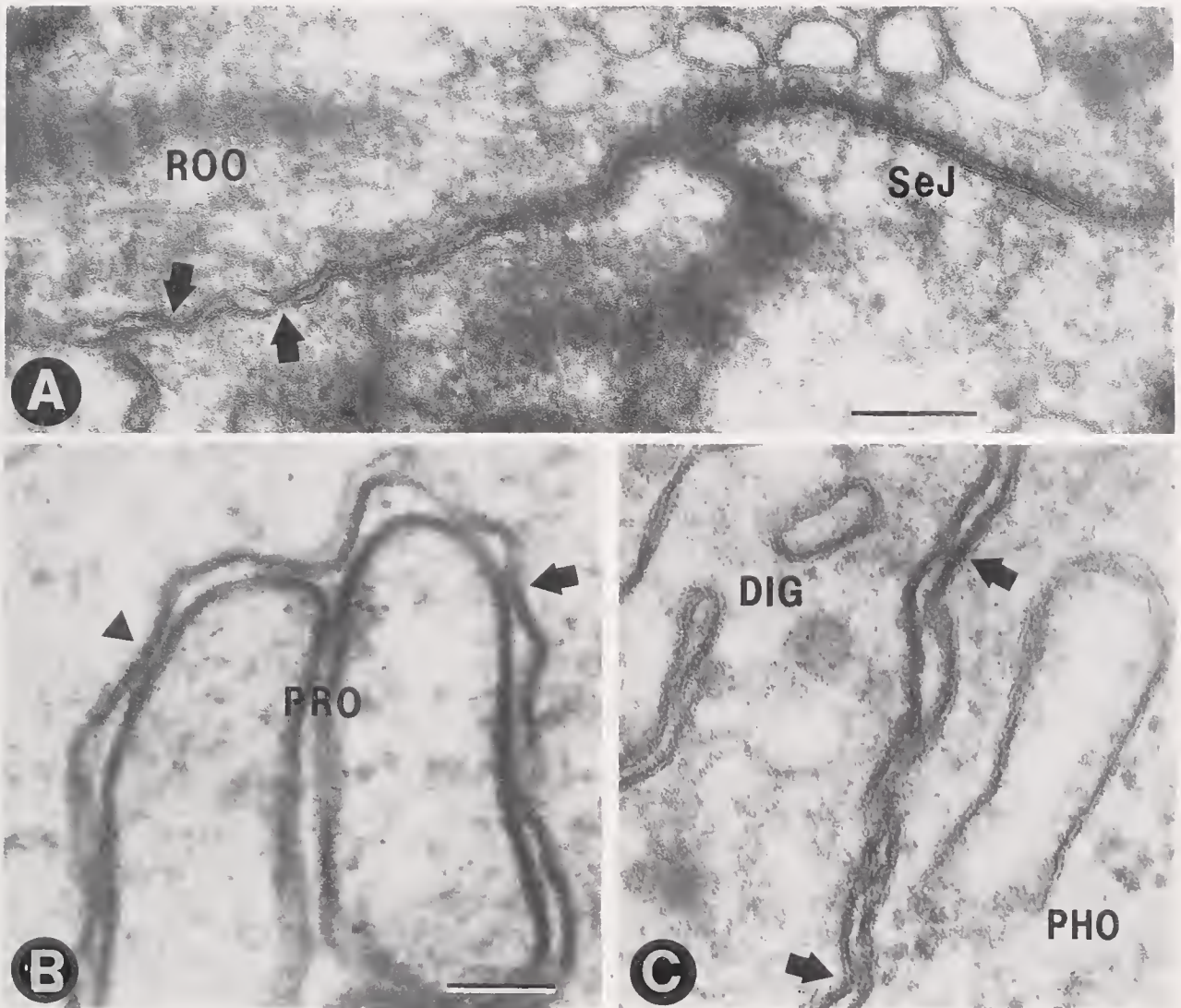


Figure 7. Electron micrographs showing small zones of close apposition (arrows) between plasma membranes of contiguous endodermal cells. (A) Between two myoepithelial cells also connected by a septate junction (SeJ); one of the cells contains a motor ciliary rootlet (ROO). (B) Between a pholocyte and two processes (PRO) of unidentified endodermal cells. Note also contact zone with intercellular gap densities displaying periodicity (arrowhead). (C) Between a digestive cell (DIG) and a pholocyte (PHO). Calibration bars = 0.2 μm (A), 0.1 μm (B,C).

belong to the vertebrate connexin family, and a case has been made for the existence of a distinct family of invertebrate gap junction proteins based on sequence homologies of *Drosophila* and *Caenorhabditis* gene products suspected of involvement in electrical synapses (Barnes, 1994). More direct evidence was recently adduced for the involvement in intercellular coupling of two such proteins derived from *Caenorhabditis* cDNA sequences and exhibiting a membrane topology strikingly similar to that of connexins (Starich *et al.*, 1996). However, the detection in *Hydra* of a protein recognized by antibodies to rat-liver gap junction proteins (Fraser *et al.*, 1987) and in

sea anemones of a mRNA hybridization signal probed with rat heart connexin cDNA (Hessinger *et al.*, 1992) lends support to the notion that the connexin family may extend phylogenetically to the phylum Cnidaria.

The Western blot detection in sea pansy tissues of a protein recognized by a monoclonal antibody to a peptide segment of rat heart Cx43 provides further evidence that a Cx43-like protein similar to that of mammals exists in anthozoans. The detection was highly specific: no other band appeared on the immunoblot except for the dimeric form of the protein, also seen in simultaneously processed blots from rat heart extracts. The peptide seg-

ment of connexin43 that served to raise the antibody is part of the cytoplasmic carboxyl terminus that presents no homology with rat liver Cx32 (Beyer *et al.*, 1987) and therefore constitutes a "signature" of that particular connexin. In contrast, that same fragment appears to have been conserved among vertebrate connexin43s. This conservation is demonstrated by the 63% identity of aligned amino acids between the corresponding sequences of the amphibian *Xenopus* and rat heart Cx43s (Gimlich *et al.*, 1990). Our results suggest that this structural conservation extends to lower invertebrates. To our knowledge, no connexin-like protein has yet been detected in invertebrates other than cnidarians; more work is thus needed to substantiate this hypothesis.

A Cx43-like protein is necessary for mediation of luminescence signaling

Use of specific antibodies raised against gap junction proteins to derive the functional role of gap junctions was pioneered by Warner *et al.* (1984) in a study of the role of gap junctions in embryogenesis. The method also proved useful in establishing a relationship between gap junction activity and developmental processes in the cnidarian *Hydra* (Fraser *et al.*, 1987). We took advantage of the detection of Cx43-like immunoreactivity in the sea pansy to use the Cx43 antibody in the same fashion and look for evidence of disruption of luminescent activity. Thus we found that the successful introduction of the antibody into sea pansy cells by permeabilization reversibly inhibited luminescent responses. The effect was not due to the permeabilization agent (DMSO) or to the antibody acting on the external surface of cells because we demonstrated that DMSO alone or the antibody delivered without DMSO did not affect luminescent responses. The possibility that the blocking effect was due to an unspecific inhibition of cellular activity by introducing macromolecules in cells was discarded when substitution of another antibody for the anti-connexin failed to block luminescent responses. Thus the Cx43 antibody effect appears to be specific and to occur from inside the cells, as predicted by the fact that the antibody recognizes a fragment of the connexin located on the cytoplasmic side (Beyer *et al.*, 1987). Although we are unaware of any previous evidence that this antibody blocks gap junction channels formed of Cx43, an antibody raised against a cytoplasmic Cx43 fragment composed of amino acids 313–324 was an effective blocker of such channels (Becker *et al.*, 1995).

The convergent results from the experiments with gap junction uncouplers and with the Cx43 antibody suggest that a Cx43-like protein is involved in mediating luminescence propagation through cell-to-cell coupling in the sea pansy. The acute inhibitory effect of the antibody on

electrically stimulated luminescence means that the antibody binds to an antigen widely distributed in the luminescent endoderm or in the nerve net that propagates the excitatory signals responsible for lighting up photocytes along the signal path (Anderson and Case, 1975).

The Cx43-like protein is localized in the luminescent endoderm and in the mesogleal nerve net

Specific Cx43-like immunoreactivity was present in all tissue layers of autozooid polyps but was more prevalent in the luminescent endoderm and in the mesogleal nerve net. Reactive sites had a predominantly punctate appearance and, in the mesogleal nerve net, a linear dotlike distribution compatible with the labeling of connexon patches (Fraser *et al.*, 1987). The higher density of reactive sites in the luminescent endoderm is not due to the presence of numerous refringent granules in cells associated with the photocytes. These granules were not immunofluorescent, and their size (1–4 μm) exceeds that of the punctate reactive sites. Thus the preferential distribution of Cx43-like immunoreactivity in this location concurs with the potent inhibitory effect of the Cx43 antibody on luminescence and suggests that a protein of the Cx43 family is an integral part of signaling among those endodermal cells. It was not possible to determine whether all endodermal cells of the luminescent zone were immunoreactive and, consequently, all potentially coupled. What is clear is that cells with refringent granules are prominent among the reactive ones. Given that the latter appear to express the β_2 -like adrenoceptors associated with luminescence control (Awad and Anctil, 1993, 1994), the likelihood is great that intercellular coupling involving Cx43-related connexons participates in downstream transmission of signals to the photocytes themselves. This arrangement would not be unique to the sea pansy— β -adrenoceptors acting *via* cyclic AMP have been implicated in the modulation of gap junction recruitment in mammalian cell lines (Radu *et al.*, 1982).

The presence of Cx43-like immunoreactivity in the mesogleal nerve net is difficult to interpret because labeling had a patchy distribution and the cellular source of labeling is unclear. Although the presence of a connexin-like protein does not necessarily confer intercellular coupling attributes, such a functional implication remains a possibility. If the amoebocytes contained a Cx43-like antigen, it would be possible to envisage a metabolic and electrical coupling function equivalent to that reported in glial cells of the mammalian CNS (Dermietzel *et al.*, 1991). If labeling is at least in part associated with neurons, the possibility of electrical coupling within the nerve net seems irreconcilable with the high magnesium sensitivity of nerve-net conduction, and interneuronal transmission *via* chemical synapses seems more likely

in anthozoans (Anderson and Case, 1975; Satterlie and Spencer, 1987). Through-conduction of mesogleal nerve-net pulses seems to imply that no interneuronal facilitation is necessary in the sea pansy (Anderson and Case, 1975). Thus coupling between cells of the mesogleal nerve net (amoebocytes and neurons) *via* connexon channels may contribute to a through-conduction feature that is otherwise difficult to account for solely on the basis of chemical neurotransmission.

Possible ultrastructural substrates of junctions involving connexons

An electron microscopic survey of polyp tissues in the sea pansy has failed to unveil any structure resembling conventional gap junctions. However, as admitted by Mackie *et al.* (1984), "the failure to observe gap junctions in these groups could merely mean that the junctions are very small, consisting of isolated connexons, or small groups of them." It was already noted above that the punctate Cx43-like immunofluorescence in the endoderm of the sea pansy was in the submicrometer range, thus substantially smaller than corresponding signals associated with true gap junction plaques (1–5 μm ; see Fraser *et al.*, 1987; Dermietzel *et al.*, 1991; Risek *et al.*, 1994). Therefore, the many small apposition zones observed at adjoining endodermal cells are possible substrates for the Cx43-like immunofluorescence. Such point contacts of limited length (50 nm or less) with a very narrow gap (2–4 nm) often containing densities may be analogous to miniature gap junctions. It is interesting that ctenophore apical zonular junctions, which are structurally similar to the sea pansy's close apposition zones, were described as minigap junctions (Hernandez-Nicaise, 1991). Thus in cnidarians, as in other metazoans, identifiable gap junctions may not be the only venue of cell-to-cell coupling, and the point contacts of close apposition described here may serve as a functional alternative using a similar constitutive membrane protein.

Acknowledgments

We thank Paul Brehm for the initial suggestion to investigate coupling in *Renilla*, Dr. Benoît Lussier for rat heart samples, and Christelle Bouchard for help and advice with electrophoresis and immunoblotting. This study was funded by grant OGP0006447 from the Natural Sciences and Engineering Research Council of Canada to M.A.

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Chemical Induction of Larval Settlement Behavior in Flow

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Abstract. The ability of dissolved chemical cues to induce larval settlement from the water column has long been debated. Through computer-assisted video motion analysis, we quantified the movements of individual oyster (*Crassostrea virginica*) larvae in a small racetrack flume at free-stream flow speeds of 2.8, 6.2, and 10.4 cm/s. In response to waterborne chemical cues, but not to seawater (control), oyster larvae moved downward in the water column and swam in slow curved paths before attaching to the flume bottom. Effective stimuli were adult-oyster-conditioned seawater (OCW) and a synthetic peptide analog (glycyl-glycyl-L-arginine) for the natural cue. The chemically mediated behavioral responses of oyster larvae in flow were essentially identical to those responses previously reported in still water. Our experimental results therefore demonstrate the capacity of waterborne cues to evoke settlement behavior in oyster pediveligers under varying hydrodynamic conditions.

Introduction

Larval recruitment is a major factor regulating the population dynamics of marine benthic invertebrates (Gaines *et al.*, 1985; Roughgarden *et al.*, 1988; Underwood and Fairweather, 1989; Menge, 1991). Two fundamental steps in the recruitment process are settlement and metamorphosis. The transport and delivery of planktonic larvae from the water column to the seabed is the settlement stage (Butman, 1987; Eckman, 1990;

Gross *et al.*, 1992). Once a substrate is accepted, larvae then metamorphose into the juvenile form (Morse *et al.*, 1979; Burke, 1983; Crisp, 1984; Bonar *et al.*, 1990).

Neither sessile nor sedentary invertebrates travel far, if at all, after metamorphosis. The ability of their larvae to select appropriate substrates is therefore critical to adult fitness (Grosberg, 1987; Levitan, 1991). Commonly, substrate discrimination is based on chemical cues associated with predators, competitors, conspecifics, or food (Burke, 1984; Woodin, 1987; Johnson and Strathmann, 1989; Morse and Morse, 1991). These cues are thought to be mostly non-waterborne and detected after larvae contact surfaces (see reviews by Morse, 1990; Pawlik, 1992).

Settlement induction by waterborne substances is often considered to be unlikely for weakly swimming invertebrate larvae (Crisp and Meadows, 1962; Butman, 1989). The locomotory speeds of these larval forms are typically lower than horizontal water-flow velocities in benthic boundary layers, even at distances of less than one larval body length above the seabed (Butman, 1986). Yet behavioral responses to chemical cues in the water column might effectively enhance larval settlement onto the substratum under some circumstances. A recent model suggests that by responding quickly to a waterborne cue, weakly swimming larvae might change their vertical distributions in the water column (making more larvae available to be swept near the substratum by fluid flow) and thus dramatically alter their patterns of settlement (Eckman *et al.*, 1994).

Oyster larvae provide a valuable tool for assessing the role of waterborne chemical cues as agents mediating settlement in flow. In assays conducted in still water, oyster

Received 16 May 1996; accepted 23 September 1996.

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(*Crassostrea virginica*) pediveligers respond to dissolved chemical cues by moving downward in the water column and attaching to the bottoms of test chambers (Tamburri *et al.*, 1992; Zimmer-Faust *et al.*, in press). The stimuli are small basic peptides (ca. 500–1,000 Da) released into seawater by adult conspecifics (Zimmer-Faust and Tamburri, 1994). A potent analog for the natural cue or cues is the tri-peptide glycyl-glycyl-L-arginine (GGR), maximally effective at 10^{-8} M.

These waterborne chemical cues may also influence oyster settlement in flowing water. In flume assays, significantly more oyster larvae accumulate on substrates in target wells releasing the synthetic peptide analog than on substrates in seawater alone (Turner *et al.*, 1994). Such distributions could result because the larvae respond to chemical cues either in the water column or after contact with test substrates. To further establish the capacity for settlement behavior in flow, we quantify the movements of oyster larvae suspended in a small race-track flume. Our results clearly demonstrate chemical induction of settlement from the water column under hydrodynamic conditions approaching those in nature.

Materials and Methods

Larval cultures

Larvae of *Crassostrea virginica* were maintained on a 12:12 D:L cycle (light on: 0700 h) at 1.0/ml in a 1:1 mixture of oceanic and artificial seawater medium (25°C and 25‰ salinity). Cultures were actively aerated and gently stirred by air bubbled through Pasteur pipettes. Marine flagellates (*Isochrysis galbana* and *Pavlova lutheri*) were provided as food at 2.5×10^4 cells/ml once each day. All experiments were begun within 6 h after 95% of the larvae in culture had developed eyes, and experiments were run for 24 h thereafter (Tamburri *et al.*, 1992). These conditions were in effect for larvae aged 19–21 days post-fertilization.

Chemical solutions

Procedures for making solutions were similar to ones previously described (Tamburri *et al.*, 1992). Both oyster-conditioned seawater (OCW) and 10^{-8} M GGR were used as test stimuli, and natural seawater (alone) was employed as control. Because a large volume of freshly prepared OCW was required in each trial, tests with this solution were limited to a speed setting of 2.8 cm/s. In contrast, trials with GGR and seawater (control) were conducted at speed settings of 2.8, 6.2, and 10.4 cm/s. Each test or control solution was prepared and filtered to $1.0 \mu\text{m}$ within 1 h before use. All experiments were conducted with solutions held at identical temperature and salinity as larval cultures.

Flume design

To minimize the number of larvae and the volume of test solutions needed per trial, all experiments were conducted in a small (40-l capacity), plastic racetrack flume (Fig. 1). The flume was 20-cm wide, consisting of two semicircular ends (20-cm radius at inner walls) and two straight sections (120-cm long). With 40 l of water, depth measured 5 cm. In one straightaway section, water flow was generated through the use of a digitally controlled motor-driven paddle wheel (48-cm total diameter) with a minimum bottom clearance of 0.3 cm. The drive wheel consisted of 12 paddles (7 cm $\times$ 19.2 cm), each tilted so that it exited vertically from the water on upstrokes. To reduce across-stream fluid motion, polycarbonate sheeting was placed parallel to the curved flume walls in the first turn to act as flow straighteners. At the downstream end of the second straightaway section, a working area was designated in which velocity profiles were taken and movements by larvae were video-recorded.

Velocity profiles were determined at each of three speed settings (2.8, 6.2, and 10.4 cm/s free-stream flow velocity, respectively) using a hot-film anemometer (TSI 1053-B). At each speed setting, a conical platinum film sensor (<1 mm diam, TSI model 1231W, 10°C over-heat) was used to measure velocity at 16 heights above the flume bottom. These data were collected at 1 Hz, using a data logger (Campbell, Model CR10). Mean flow speed at each setting and height was determined by averaging measurements over 1-min intervals. To establish the shear velocity (U_*) for flow at each speed setting, mean flow velocities were regressed against the natural logarithm of height above the flume bottom (Nowell *et al.*, 1981; Jonsson *et al.*, 1991).

Experimental protocol

Separate trials were performed in the flume with either OCW, GGR, or seawater (control) at a uniform concentration mixed throughout the water column. In each trial, we suspended 2000 ± 358 (range) larvae in a beaker with 200–300 ml of seawater. The larvae were then gently poured from the beaker (over 5–10 s) into the flume at a site raised about 4 cm above the bottom and 1.0 m upstream of the working section. Video-recording was limited to the first pass of larvae over the working section. The flume was drained and then cleaned with several rinses of distilled water between each trial. Six trials were run for each solution at the two slowest speed settings, and three trials each at the fastest speed.

Video imaging and motion analysis

Simultaneous video-recordings from two cameras (Panasonic WV-BL-600), each equipped with a 55-mm

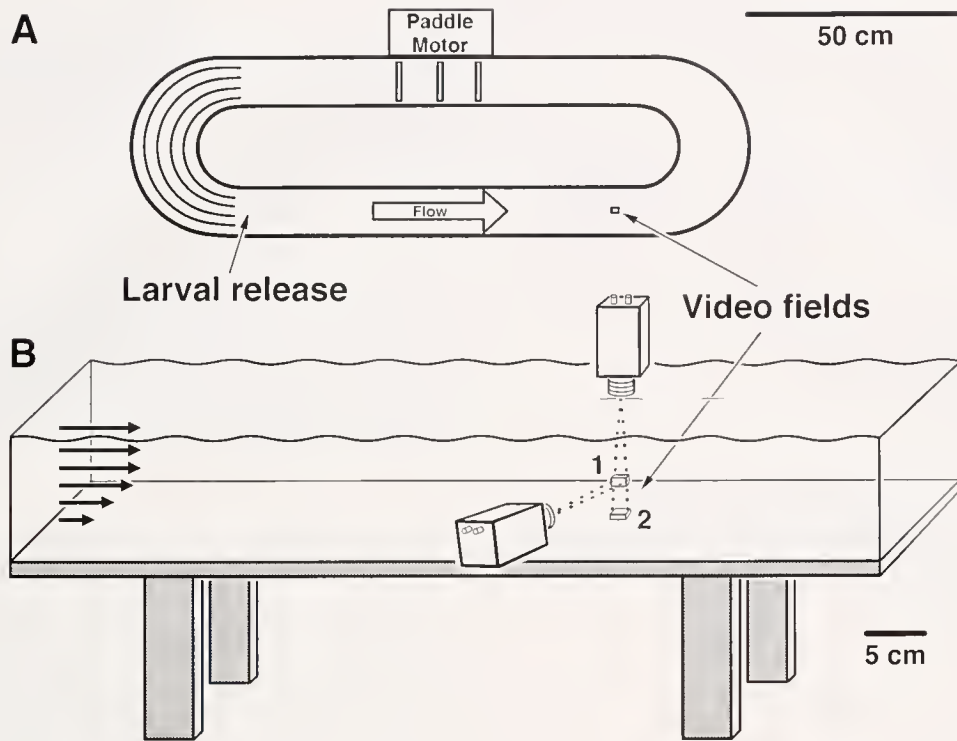


Figure 1. Schematics showing the small racetrack flume used in this study. In each trial, the flume was filled with 40 l of either oyster-conditioned seawater (OCW) or glycyl-glycyl-L-arginine (GGR) at a uniform concentration, or with seawater control. Following their release, larvae were video-recorded as they passed for the first time over the working area. (A) Top view indicating the direction of water flow, position of the paddlewheel, site at which larvae were released, and position and size of the working area in which video records were made. (B) A 3-dimensional view more closely indicating the video fields viewed by each of our two cameras. 1 = vertical field in the water column as viewed by the side-mounted camera. 2 = horizontal field in the bottom boundary layer as viewed by the overhead-mounted camera. Each field was 1.2-cm long and 0.7-cm wide, with a focal depth of 0.4 cm.

macro-lens, were made as larvae passed through the working section. One camera was mounted above and the other to the side of the flume (Fig. 1). Outputs from both cameras were run through a video splitter (American Dynamics, model AD1470A), recorded onto a single tape, and viewed on a monitor. The entire working section was illuminated with dim, far-red light (>650 nm). The side-mounted camera was positioned to view larvae in the free-stream, a minimum of 1.9 cm above the flume bottom. In contrast, the overhead camera was placed to view larvae within the boundary layer at heights between 0 and 4 mm above the bottom. By using a 4-mm focal depth in video-recordings, individual larvae could be tracked over the entire length of each video field. The field size recorded by each camera was 1.2×0.7 cm with the long axis parallel to the direction of water flow. Estimates of settlement activity were, therefore, made only for those larvae in the video field.

Methods for quantifying larval movements were identical to those previously described (Tamburri *et al.*,

1992; Tamburri and Zimmer-Faust, 1996). Video records were processed at 30 frames/s through a motion analyzer (Motion Analysis Corp. model VP 110 and ExpertVision software) interfaced with a Sun Microsystems SPARC IPC computer workstation. Because of the small video field (1.2×0.7 cm), both vertical orientation and speed at which larvae moved either upward or downward in the water column could be accurately determined only in the slowest flow (2.8 cm/s). Vertical motion was measured as net trajectory (change in vertical position divided by change in horizontal position between the beginning and end points of each path). In contrast, the number of larvae attaching to the bottom (herein defined as *settlers*), and both horizontal velocity and angular velocity (measured as rate of change in direction, RCDI) could be accurately quantified for movements by larvae in flows at all three speed settings. Our analyses of horizontal speed and RCDI were limited to those paths in which larvae did not contact the bottom.

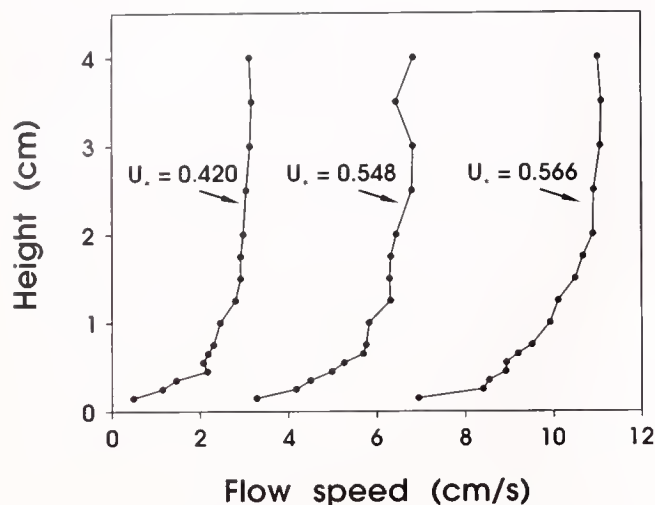


Figure 2. Velocity profiles for flowing water at each of the three flume settings used in experiments. From left to right, each profile is for flow with a free-stream speed of 2.8, 6.2, or 10.4 cm/s; shear velocity (U_*) for each flow is shown.

Results

Hydrodynamics

Velocity profiles, as well as shear velocity (U_*), for flow at each speed setting are shown in Figure 2. The average velocity ($\bar{U}$) for the slowest flow was 2.75 cm/s, for the intermediate flow was 6.21 cm/s, and for the fastest flow was 10.35 cm/s. Regressions used to calculate shear velocities accounted for more than 95% of the variation in the data in each case.

Settlement and behavior of larvae in flow

The behavior of oyster larvae in response to seawater (control) was markedly different from that in response to OCW and GGR (Kruskal-Wallis $\chi^2 = 12.74$, $df = 2$, $P = 0.002$). In seawater, the average trajectory was upward (2.9% slope), indicating that the larvae remain suspended above the flume bottom (Table I). An opposite effect was found in response to OCW and GGR—the average trajectory was downward (3.4% and 3.8% slope, Table I). Mean speeds at which downward-directed larvae moved in response to OCW and GGR were 1.7 mm/s (± 0.6 mm/s SEM) and 1.8 mm/s (± 0.7 mm/s SEM), respectively. After traveling downward in the water column, larvae approached the flume bottom and settled. Hence, the numbers of settlers in OCW at 2.8 cm/s flow speed and in GGR at 2.8 and 6.2 cm/s were significantly higher than those in seawater (control) at the same flow speeds (Table II, Kruskal-Wallis test). Although not statistically significant, there was a trend in the same direction for GGR at the fastest flow speed (10.4 cm/s, Table II).

Table I

Mean ($\pm$ SEM) trajectory calculated as percent slope for each larval path in response to test or control solution at 2.8 cm/s flow speed

Solution	n	% Slope	Significance
SWC	39	2.93 ± 1.51	A
GGR	43	-3.80 ± 1.81	B
OCW	32	-3.42 ± 1.37	B

SWC = seawater control, GGR = 10^{-8} M glycyl-glycyl-L-arginine, OCW = adult-oyster-conditioned seawater. Trajectories were calculated as the ratio of change in vertical position to change in horizontal position between beginning and end points of paths. Positive values represent net upward motion and negative values represent net downward motion. Groups with different letters indicate a statistical difference at the $\alpha = 0.05/3 = 0.017$ level.

The exact height at which each larva swam within 4 mm of the flume bottom could not be precisely identified. Because the speed of fluid motion decreased close to the bottom (Fig. 2), determinations of velocity and rate of change in direction (RCDI) for horizontal movements by larvae reflected components of both active swimming and passive flow. We therefore made comparisons between test and control treatments separately at each speed setting. In this context, slower larval velocity indicates that a larva either swims closer to the flume bottom or indeed swims more slowly. In either case, a significant difference between stimulus and control treatments demonstrates a significant change in larval behavior. With these caveats in mind, we found that paths traveled by larvae in seawater (control) were significantly straighter (lower mean RCDI) and faster than those in OCW and GGR (Table III, Kruskal-Wallis $P < 0.05$ experimental-wise error, all comparisons). Remarkably, larvae often turned and swam upstream in response to GGR at 2.8 and 6.2 cm/s and to OCW at 2.8 cm/s (the only flow speed at which this solution was tested), but never in response to seawater (Fig. 3).

Table II

Mean ($\pm$ SEM) number of larvae settling on the flume bottom in response to either test or control solution

Solution	Flow speed (cm/s)		
	2.8	6.2	10.4
SWC	1.00 ± 0.37 A	0.83 ± 0.31 A	1.67 ± 0.67 A
GGR	6.00 ± 1.84 B	6.00 ± 0.82 B	3.67 ± 0.88 A
OCW	4.50 ± 0.85 B	Not determined	Not determined

SWC = seawater control, GGR = 10^{-8} M glycyl-glycyl-L-arginine, OCW = adult-oyster-conditioned seawater. Groups with different letters indicate a statistical difference at the $\alpha = 0.05/3 = 0.017$ level using the Kruskal-Wallis chi square test.

Table III

Mean ($\pm$ SEM) speed and rate of change in direction (RCDI) for horizontal movements by larvae, and number of larvae turning upstream in response to either test or control solution

Flow speed (cm/s)	Solution	Movement by larvae		Number of larvae	
		Speed (mm/s)	RCDI (degrees/s)	Observed	Moving upstream
2.8	SWC	1.69 $\pm$ 0.18 A	25.2 $\pm$ 4.0 A	2.5 $\pm$ 0.5	0
	GGR	0.49 $\pm$ 0.14 B	122.4 $\pm$ 20.6 B	3.2 $\pm$ 0.5	1.7 $\pm$ 0.6
	OCW	0.97 $\pm$ 0.21 B	93.7 $\pm$ 20.8 B	3.2 $\pm$ 0.5	1.2 $\pm$ 0.3
6.2	SWC	3.91 $\pm$ 0.41 A	22.4 $\pm$ 4.1 A	2.8 $\pm$ 0.4	0
	GGR	1.88 $\pm$ 0.38 B	81.0 $\pm$ 15.1 B	3.7 $\pm$ 0.5	0.4 $\pm$ 0.2
10.4	SWC	9.31 $\pm$ 0.55 A	32.9 $\pm$ 6.5 A	2.3 $\pm$ 0.3	0
	GGR	6.64 $\pm$ 0.60 B	155.6 $\pm$ 57.4 B	3.7 $\pm$ 0.3	0

SWC = seawater control, GGR = 10^{-8} M glycyl-glycyl-L-arginine, OCW = adult-oyster-conditioned seawater. Speed and RCDI were limited to those paths in which larvae did not contact the bottom, whereas all paths in the field of view were examined for movement upstream. Groups with different letters indicate a statistical difference at the $\alpha = 0.05/3 = 0.017$ level using the Kruskal-Wallis chi square test.

Discussion

These results show that waterborne chemical cues evoke settlement in flow by planktonic oyster larvae. Pediveligers responded to dissolved compounds by moving downward in the water column, then swimming in slow curved paths and attaching to the flume bottom. The behavior we observed in flume flows was essentially identical to that previously described in still water (Tamburri *et al.*, 1992; Zimmer-Faust *et al.*, in press). Whereas still-water assays are valuable in describing behavioral responses, both flume and field experiments are required to identify those hydrodynamic environments in which such behavior allows larvae to select settlement sites.

Important insights to settlement processes can be gained by considering the relationship between hydrodynamics and larvae swimming and tumbling in bottom boundary-layer flows (Jonsson *et al.*, 1991; Mullineaux and Butman, 1991; Pawlik *et al.*, 1991; Grassle *et al.*, 1992; Pawlik and Butman, 1993). When flow speeds and shear velocities were below critical thresholds at which larvae become transported as bed load (about 4–5 cm/s and 0.25 cm/s, respectively), in the absence of waterborne chemical cues, weakly swimming cockle (*Cerastoderma edule*, Jonsson *et al.*, 1991), worm (*Phragmatopoma lapidosa californica*, Pawlik and Butman, 1993), and oyster larvae (this study) all propelled themselves upward to remain suspended in the water column. This behavioral response allows the larvae to avoid settling in relatively slow flows even though physical attachment is possible (Pawlik and Butman, 1993). At faster flow speeds and higher shear velocities, where bed-load transport occurs but resuspension is rare, both cockle and worm larvae become trapped in the near-bed flow and roll along the bottom (Jonsson *et al.*, 1991; Pawlik

and Butman, 1993). Perhaps we did not observe rolling for oyster pediveligers because they were introduced at 4 cm above the bottom. At flow speeds of 6.2 and 10.4 cm/s, oyster larvae would have had only about 10–20 s after introduction to either sink or swim downward before being swept over the working section of the flume. Indeed, visual observations of oyster larvae moving downstream in a larger flume (4.8-m long) previously indicated that larvae were always close (<2 mm) to the bed (Turner *et al.*, 1994). If the hydrodynamic regime confines larvae close to the bed (Jonsson *et al.*, 1991), larvae will not need to settle very far to contact the bottom. Even a small change in vertical movement caused by a waterborne chemical cue might thus be important in mediating settlement under some flow conditions.

The Rouse number reflects the strength of the downward transport of larvae due to gravitational sinking and swimming, relative to the upward transport of larvae due to turbulent mixing (Gross *et al.*, 1992; Eckman *et al.*, 1994). This number is a dimensionless ratio (w_j/kU_*), where w_j is downward transport due to larval sinking and swimming, k is von Karman's constant (=0.41), and U_* is shear velocity. At Rouse numbers greater than 0.75, larval swimming and sinking can have a substantial impact on settlement from a turbulent, bottom boundary layer (Gross *et al.*, 1992). In response to a waterborne chemical cue, oyster pediveligers move downward at average and maximum speeds of 1.8 mm/s and 3.1 mm/s, respectively (Turner *et al.*, 1994; this study). Furthermore, shear velocity of water flowing over an oyster reef scales at 0.17-times the free-stream speed (Wright *et al.*, 1990). We observed a Rouse number greater than 0.75 for flow speeds below 3.5 cm/s, indicating that settlement is not inhibited by turbulence. At flow speeds of 6 cm/s, only the most rapidly swimming larvae (vertical

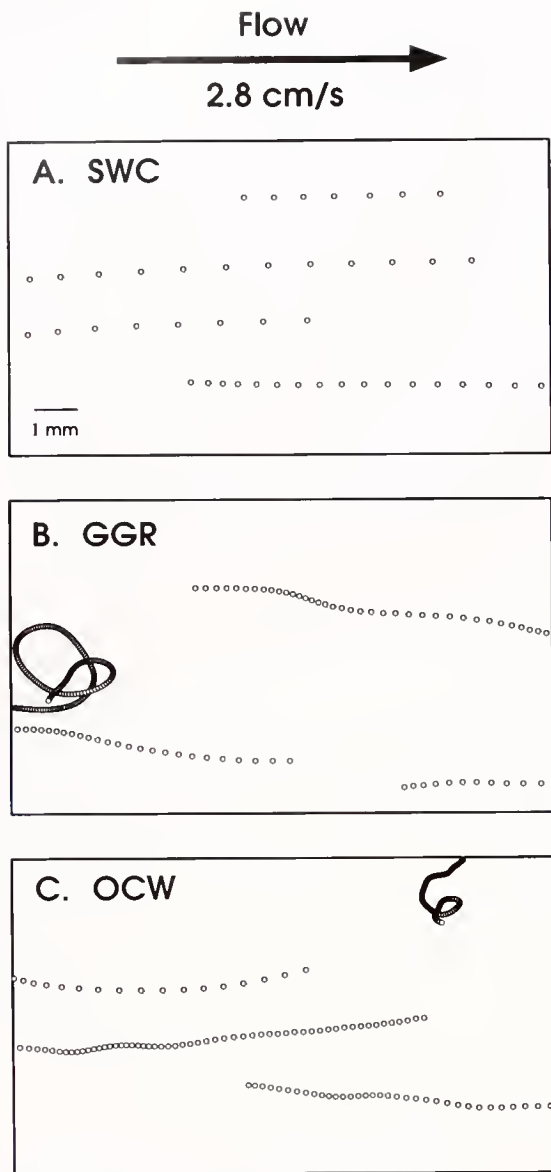


Figure 3. Computer-digitized video records showing the horizontal paths of larvae moving within 4 mm of the flume bottom and entering the 1.2- × 0.7-cm field viewed by the overhead camera. The paths generated for four larvae appear in each box. Each path of dots presents the position of a larva at consecutive, one-frame intervals with video collected at 30 frames/s. We selected the paths to illustrate the various movement patterns (including upstream swimming) observed in flowing water at 2.8 cm/s. These data are for larvae exposed to either (A) seawater control (SWC), (B) 10^{-8} M glycyl-glycyl-L-arginine (GGR), or (C) oyster-conditioned seawater (OCW). Paths are straighter and faster in response to SWC than to either GGR or OCW.

velocities > 3 mm/s) can overcome turbulent transport. Remarkably, these speeds are common in water flowing above oyster reefs in tidal estuaries (Breitburg *et al.*, 1995).

A model for benthic-habitat site selection by weakly

swimming invertebrate larvae has been proposed by Butman and Grassle (1992). In this model, larvae are hypothesized to move up and down in the water column close to the bottom, while being transported by flow, and to test substrates on contact. Selection is thus accomplished by active acceptance or rejection of touchdown sites. We suggest, at least for oyster pediveligers, that behavioral response to a waterborne chemical cue is an additional factor in habitat selection. Oyster larvae rapidly respond (within 5 s of initial exposure) to dissolved substances produced by juvenile and adult conspecifics and by bacteria in biofilms on the surface of oyster shell (Tamburri *et al.*, 1992; Zimmer-Faust *et al.*, in press). These stimulatory compounds are low molecular weight peptides with arginine at the C-terminal; at concentrations of 10^{-10} M, or lower, they effectively cause downward-directed movements by oyster larvae in the water column (Zimmer-Faust and Tamburri, 1994). Because this behavioral response does not require a chemical gradient, induction of settlement should be possible even in turbulent-flow environments. Presumably, downward movements increase the probability that oyster larvae will be swept into contact with preferred benthic substrata by near-bed turbulent eddies.

Juvenile and adult oysters commonly form extensive aggregations (10,000 m², and beyond) at high densities (15,000 individuals/m²) in benthic estuarine habitats (Bahr, 1981). Waterborne compounds produced by post-metamorphic oysters will thus be distributed over large areas, rather than confined to isolated point sources. Field data further indicate that flow velocities measured 2–10 cm above oyster reefs rarely exceed 7 cm/s (Breitburg *et al.*, 1995). This means that oyster larvae drifting near the bottom will likely pass over cued substratum for several minutes, long enough for the induction of a settlement response.

We suggest that the effectiveness of waterborne settlement cues may be greatest in environments similar to those in which oyster larvae settle. Perhaps the capacity of dissolved compounds to influence settlement is linked to cue production and, therefore, is most important in habitats that have large aggregations of post-metamorphic conspecifics or food resources. Waterborne cues might also function as general chemical signatures indicating the presence of preferred substrates, such as those provided within coral reefs, kelp forests, and organic-rich mud flats. Although the effects of waterborne chemical cues may be profound, the extent to which such cues influence settlement remains largely unexplored.

Acknowledgments

This research was sponsored by awards from the National Science Foundation (OCE 94-16749), South Car-

olina Sea Grant Consortium (P-M-2F, P-M-2K, R-92-88), Office of Naval Research (N0014-K-82-646), Slo-cum-Lunz Foundation, Lerner-Gray Fund for Marine Research, and Sigma Xi. We thank K. Kurkowski and V. L. Shaffer for generously providing larvae, C. C. Gee for assistance in illustrating larval paths, and N. D. Pent-cheff and Drs. A. W. Decho, M. W. Luckenbach, and S. E. Stancyk for their comments, which greatly im-proved earlier drafts of this manuscript.

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Morphological Evidence for a Chitin-Like Glycoprotein in Penaeid Hatching Envelopes

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Abstract. When chitin hydrolytic enzymes (chitinase and *N*-acetylglucosaminidase) were used as treatment during formation of the hatching envelope (HE) of the penaeid shrimp *Sicyonia ingentis*, results indicated the presence of carbohydrate moieties in the envelope. Eggs exposed to these enzymes had abnormal HEs that might elevate and often collapsed. The finding that chitin synthase inhibitors (tunicamycin, nikkomycin Z, and polyoxin D) also interfered with normal HE formation is further evidence for a carbohydrate component. The application of these synthase inhibitors resulted in more fragile envelopes that elevated and collapsed or were easily lost during processing. Similar results were seen in the absence of divalent ions (magnesium and calcium) considered critical for normal chitin formation. This morphological evidence is indicative of a chitin-like, linked carbohydrate in the HE of *Sicyonia ingentis*.

Introduction

Oocyte activation in the marine rock shrimp *Sicyonia ingentis* takes place upon contact with seawater and is independent of fertilization (Pillai and Clark, 1987). The

appearance of a primary extracellular coat around the "egg" is usually associated with activation of the oocyte. The components of this extracellular, or extra-embryonic, coat determine, in part, the resistance of the embryo to invasion by pathogens and establish a micro-environment for the developing zygote. In *S. ingentis* eggs, this extracellular coat appears about 45 min after spawning (Pillai and Clark, 1987, 1988). The presence of carbohydrate linkages in the extracellular coat of the crustacean egg is suggested by the abundance of saccharides in hatching envelopes (HE) isolated from shrimp (Pillai and Clark, 1990), by lectin labeling in shrimp HE (Pillai and Clark, 1990), and by the localization of *N*-acetylglucosamine in the copepod fertilization envelope (Santella, 1993). Pillai and Clark (1990) demonstrated carbohydrate components, in particular, *N*-acetylglucosamine and mannose, in *Sicyonia ingentis* HE.

We postulate that at least three enzymes are necessary for assembly of the HE in penaeid shrimp eggs: an oxidase (Glas *et al.*, 1995), a transaminase (Glas *et al.*, 1992), and a carbohydrate synthase (Glas *et al.*, 1993). One of the most abundant carbohydrate components of decapod crustaceans that require a carbohydrate synthase is the polymer of β -1,4-*N*-acetylglucosamine, more commonly known as chitin. In this work, we show that enzymes that hydrolyze chitin or inhibit chitin synthase change HE elevation and assembly. In addition, chitin formation is reported to be dependent on ion concentration and composition (Warner, 1977; Stevenson, 1985). Clark and Lynn (1977) and Pillai and Clark (1987) reported that calcium and magnesium ions are necessary for the initial elevation of penaeid egg HE. In this study, we used ion-deficient seawater to determine the longer term effects of divalent ions on HE assembly and elevation in *S. ingentis* eggs. These morphological changes

Received 2 April 1996; accepted 4 September 1996.

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Abbreviations: hatching envelope, HE; ethylenediaminetetraacetic acid, EDTA; ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid, EGTA; transmission electron microscopy, TEM; fluorescein isothiocyanate, FITC; tetramethylrhodamine isothiocyanate, TRITC.

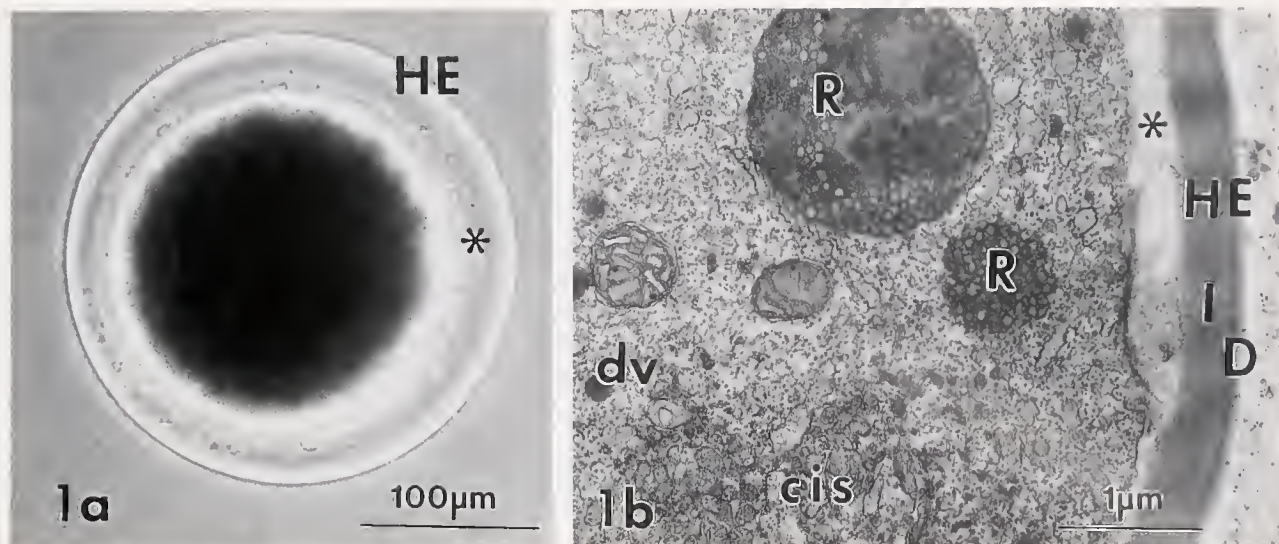


Figure 1. Eggs of *Sicyonia ingentis* 90 min postspawn in artificial seawater. (a) Eggs spawned into artificial seawater elevated a hatching envelope (HE) with a distinct perivitelline space containing a refractile material. Bar equals 100 μm . (b) Eggs spawned into seawater elevated a bilayered HE with a dense outer layer and a less dense inner layer. Bar equals 1 μm . HE, hatching envelope; D, dense layer; I, inner layer; *, perivitelline space; R, ring vesicles; dv, dense vesicles; cis, endoplasmic reticulum cisternae.

offer evidence of a chitin-like carbohydrate linkage in penaeid HE.

Materials and Methods

Gamete collection and handling

The rock shrimp *Sicyonia ingentis* was collected by otter trawl from waters off the southern coast of California adjacent to Santa Barbara and Los Angeles. Animals were transported in cooled, aerated tanks to Bodega Marine Laboratory, Bodega Bay, California, where they were maintained with constant light in flow-through aquaria. Spawning was induced by placing the females in darkness according to the methods of Pillai *et al.* (1988). Spawning females were held over 50 $\times$ 70 mm crystalizing dishes filled with artificial seawater at room temperature (20°C) to collect the eggs. Artificial seawater was prepared according to Cavanaugh (1956), with salinity adjusted to 30‰. Controls for each experiment were maintained in the artificial seawater at room temperature ($\sim$ 22°C) during the experiments.

Inhibitors of fungal chitin synthesis, tunicamycin, nikkomycin Z, and polyoxin D (Calbiochem), were added to the eggs in seawater (pH 8.0) to give a final concentration of 1 μM . Chitin hydrolytic enzymes, chitinase (1 $\mu\text{g}/\text{ml}$ or 1.5×10^{-4} units) (Sigma, C-1525) and *N*-acetylglucosaminidase (0.05 units/ml) (Sigma, A-3189), were added to eggs in seawater at 10 min postspawn. Samples

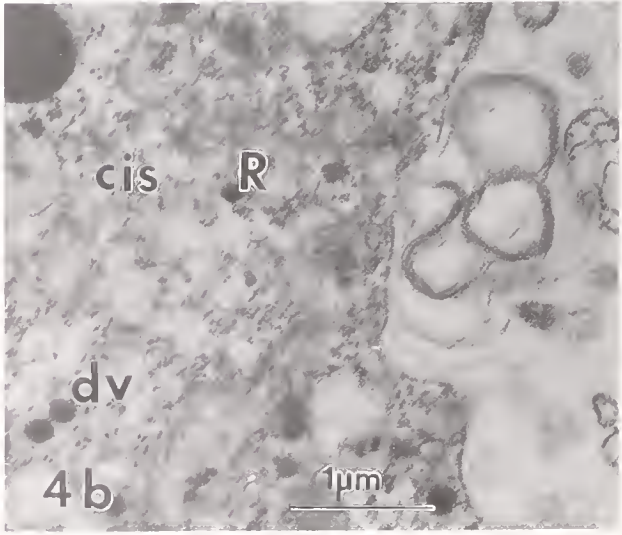
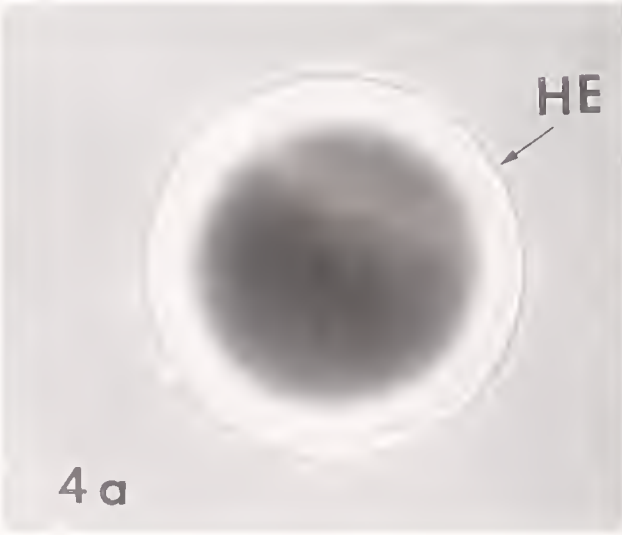
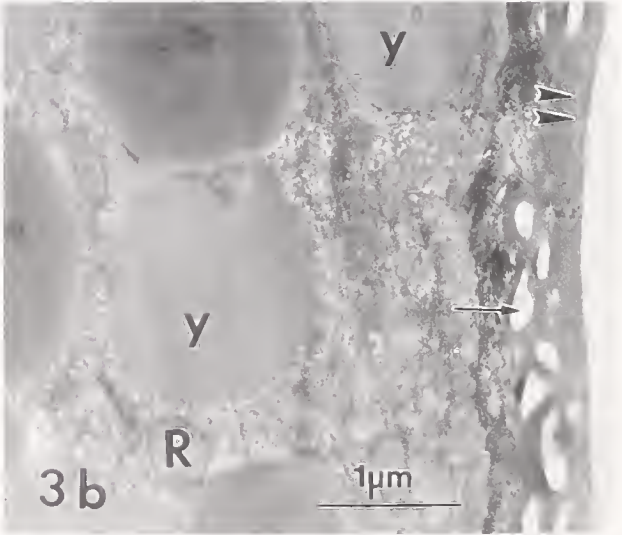
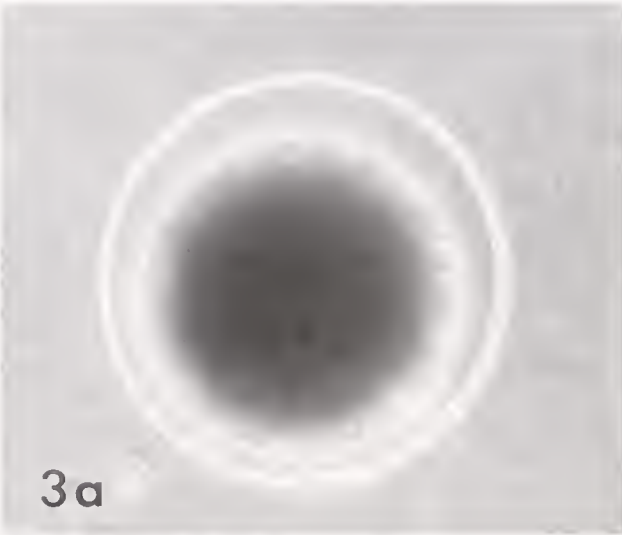
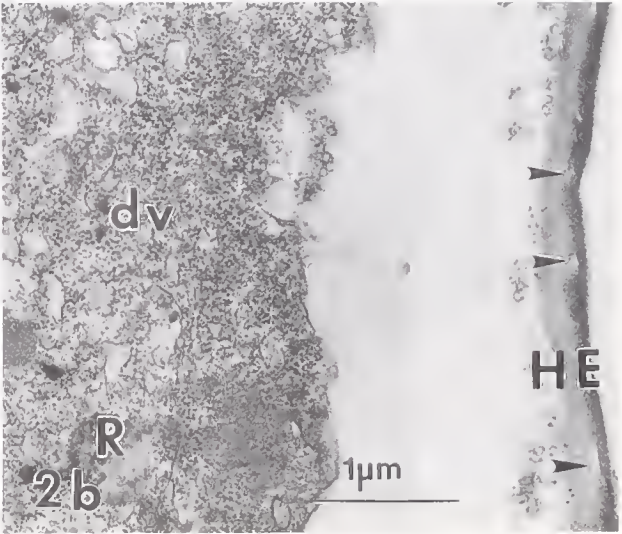
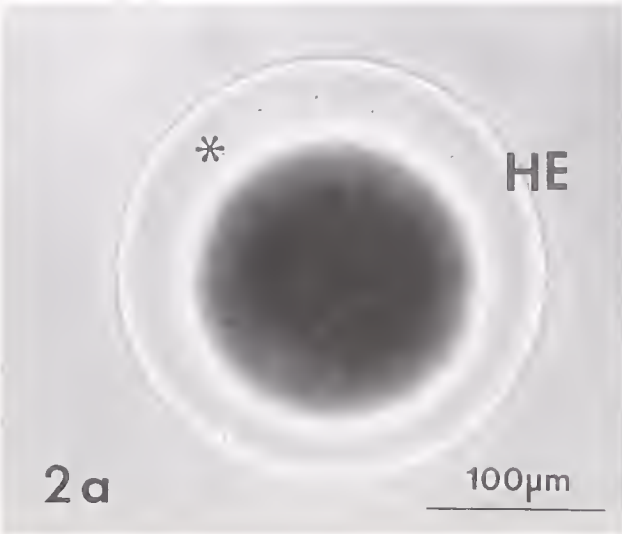
were removed for microscopic examination at 90 min postspawn—*i.e.*, well after normal formation of the HE, a fertilization-independent event (Pillai and Clark, 1987).

To observe the effects of ion dependency on HE assembly, seawaters deficient in calcium ions, magnesium ions, or both were used. Chelator concentrations of 5 mM EDTA (for calcium and magnesium) or 2 mM EGTA (for calcium) were added to the ion-deficient seawater to bind extraneous calcium and magnesium ions. The seawater was adjusted with sodium salts to maintain osmolarity. For ion-deficiency experiments, eggs were spawned into artificial seawater, and after 10 min the water was changed three times with the appropriate ion-deficient seawater. Thus, interference with early, ion-dependent events in egg activation was avoided (Clark and Lynn, 1977; Lindsay *et al.*, 1992).

Microscopy

Aliquots of eggs from the spawning dish were placed on clean glass slides with coverslips supported by silicon grease to prevent desiccation and mechanical disruption of the eggs. Fresh aliquots were periodically prepared from the appropriate spawning dishes. Eggs were observed and photographed with phase microscopy during each experiment.

Samples for histology and electron microscopy were removed at 90 min postspawn and fixed with 1.2% para-



formaldehyde and 0.8% glutaraldehyde in 0.28 M sodium cacodylate buffer in 33% seawater, pH 8.3, for 1 h at room temperature.

Electron microscopy

Samples for transmission electron microscopy (TEM) were washed three times in 0.4 M cacodylate buffer and post-fixed with a final concentration of 1% osmium tetroxide in 0.2 M cacodylate buffer for 45 min at room temperature. Samples were then rinsed, dehydrated through a graded acetone series, and embedded in a modified Spurr's resin (Spurr, 1969), substituting Quetol 651 for DER 736 resin. Thin sections were cut with a diamond knife, floated on copper grids, double stained with lead citrate (Venable and Coggeshall, 1965) and methanolic uranyl acetate, and viewed by TEM.

Hatching envelope isolation and lectin binding

Hatching envelopes from *S. ingentis* eggs 90 min postspawn were isolated according to the method of Pillai and Clark (1990) and Wikramanyake and Clark (1994). The eggs were homogenized in a Potter-Elvehjem homogenizer in 0.5 M NaCl with 5 mM benzamidine hydrochloride and 0.1% Nonidet (Sigma Chemical Co.). For the homogenization, the solution was dropped to pH 5 with a few drops of HCl to help with removal of the lipid. Subsequent washes were at pH 7.8. The homogenate was diluted 10 times with the homogenizing solution and centrifuged at $200 \times g$ for 5 min. The pellet was washed repeatedly until translucent, then stored in liquid nitrogen.

The frozen samples were thawed and lectin-binding assays on envelopes from the same isolation procedure were carried out followed the methods of Kiernan (1990) and Pillai and Clark (1990). Lectins labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC), both at 50 $\mu\text{g}/\text{ml}$, were used to

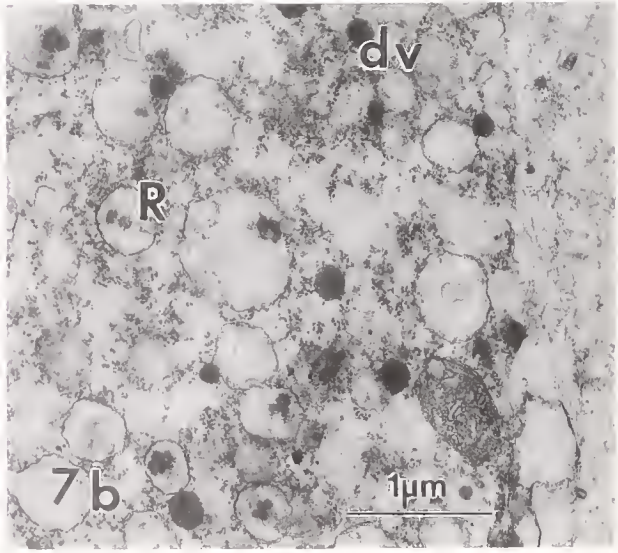
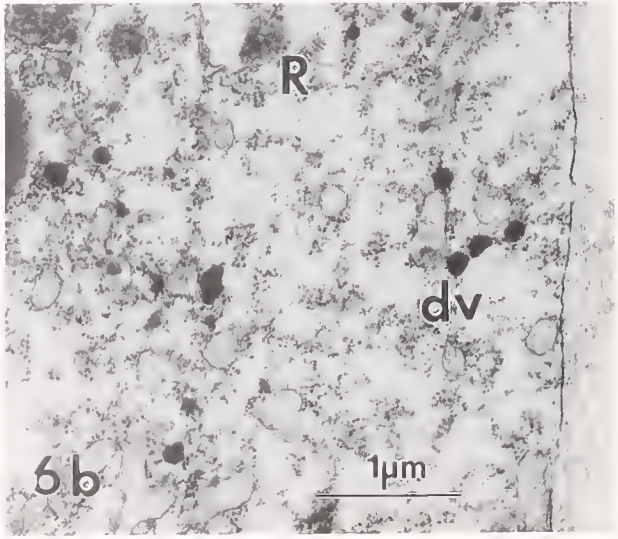
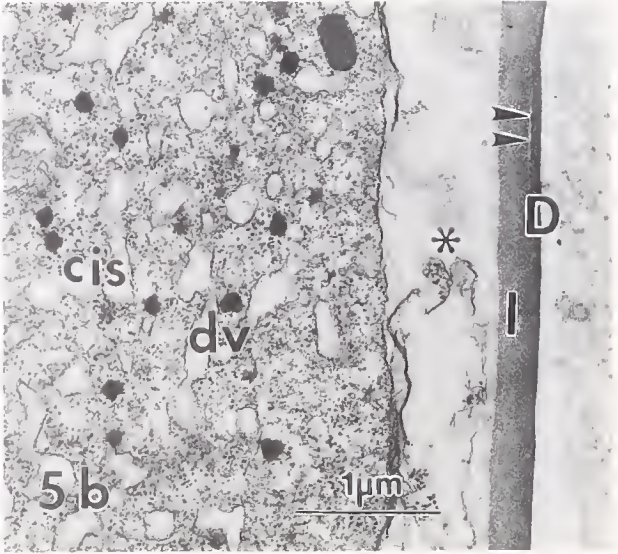
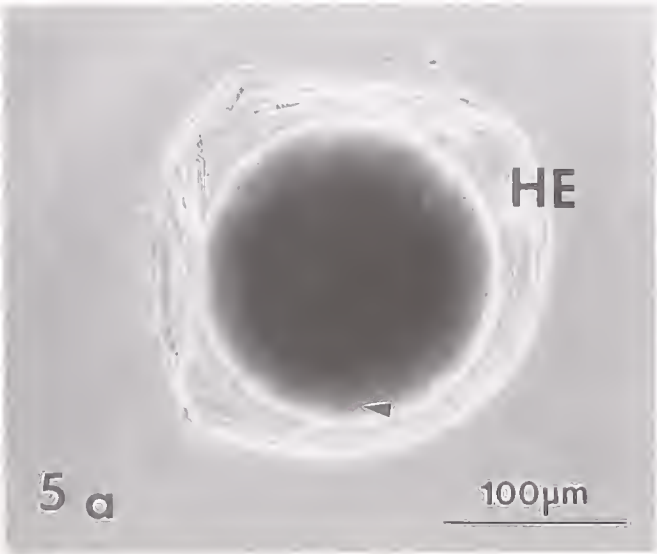
identify terminal sugars. Concanavalin A (Con A) for mannose and glucose, wheat germ agglutinin (WGA) for *N*-acetylglucosamine and sialic acid, *Lens culinaris* agglutinin (LCA) for mannose, and *Bandeirea simplicifolia* (BS-II) for *N*-acetylglucosamine (all lectins from Sigma Chemicals) were applied in a 0.1 M phosphate buffer, pH 7.2, with trace metals added. Control samples contained 200 mM solutions of the appropriate blocking sugars (mannose, glucose, and *N*-acetylglucosamine) preincubated with the lectin for 10 min prior to application to the section (Kiernan, 1990; Pillai and Clark, 1990). Isolated HEs were incubated in the presence of the fluorescently labeled lectins for 1 h, then rinsed three times in phosphate buffer. Samples were observed with an epifluorescent microscope equipped with dichroic filter blocks for FITC and TRITC.

Results

Spawned eggs were observed for normal HE assembly and elevation in the presence or absence of chitin synthase inhibitors, chitin hydrolytic enzymes, and divalent ions. Criteria for normal elevation were the continued separation of the HE from the oolemma, as viewed by light microscopy, and the presence of the bilayered structure indicative of normal HE assembly, as described by Pillai and Clark (1988).

The control eggs began elevating the HE 40–50 min postspawn and showed normal HE formation at 60 min postspawn (Pillai and Clark, 1987, 1988). By 90 min postspawn, a refractile HE was formed around the egg (Fig. 1a). The perivitelline space contained a refractile material that is believed to add to the HE until the next extra-embryonic envelope is formed. Viewed with TEM, the HE had a thin, electron-dense outer layer and a thicker, less electron-dense inner layer (Fig. 1b) formed initially from components of the ring vesicles, as described by Pillai and Clark (1988). The cortex of the egg still contained many small, electron-dense vesicles and

Figures 2–4. Eggs of *Sicyonia ingentis* treated with chitin synthase inhibitors elevate an abnormal hatching envelope (HE). (2a) Eggs treated with tunicamycin sometimes appeared normal, with an elevated HE. The material in the perivitelline space looked finer than in the control eggs. Bar equals 100 μm . (2b) When viewed with TEM, the HE around eggs treated with tunicamycin appeared similar to the control eggs, but there are "holes" between the HE layers (arrowhead). The micrograph is in two sections to allow a greater view of the cortex of the egg. Bar equals 1 μm . (3a) Eggs treated with polyoxin D elevated envelopes that appeared to have a normal HE. (3b) When viewed with TEM, eggs in polyoxin D had abnormal HEs with clear spaces or "holes" in the inner layer (arrow) and between the two envelope layers (arrowhead). Large yolk granules (y) displaced the ring vesicles and mitochondria to the cortex of the egg. (4a) Eggs treated with nikkomycin Z elevated HEs (arrowhead) that remained close to the egg. (4b) Eggs in nikkomycin Z viewed with TEM appeared to have lost the HEs in the fixation and embedding process. The cisternae and dense vesicles were situated normally, but the ring vesicles (R) were not as packed as in the controls. HE, hatching envelope; D, dense layer; I, inner layer; *, perivitelline space; R, ring vesicles; dv, dense vesicles; cis, endoplasmic reticulum cisternae; y, yolk platelets.



large ring vesicles tightly packed with ring-shaped elements. These vesicles appeared similar to those observed by Pillai and Clark (1988) during the early formation of the HE. Small cisternae of endoplasmic reticulum (ER) occupied much of the cortical region (Fig. 1b). Vesicles loosely filled with ring elements and mitochondria in close association with Golgi complexes were frequently observed. For a more detailed description of early HE formation, refer to Pillai and Clark (1988, 1990).

Chitin synthesis inhibitors block normal HE formation

Eggs were treated with three chitin synthase inhibitors, tunicamycin, nikkomycin Z, and polyoxin D. In the treated eggs, HE formation, elevation, or both were abnormal. Eggs treated with tunicamycin elevated HE in two out of three trials when assayed with light microscopy (Fig. 2a). In some eggs, the HE remained close to the egg surface so that the perivitelline space was obscured (not shown). Granular material finer than that observed in control eggs was seen in the perivitelline space. Eggs treated with polyoxin D had envelopes that appeared similar in structure to the control egg when viewed with light microscopy (Fig. 3a). In eggs treated with nikkomycin Z, the HE remained close to the egg surface (Fig. 4a) and the perivitelline space was filled with a fine material, often making it difficult to visualize the separation from the egg surface when viewed with light microscopy.

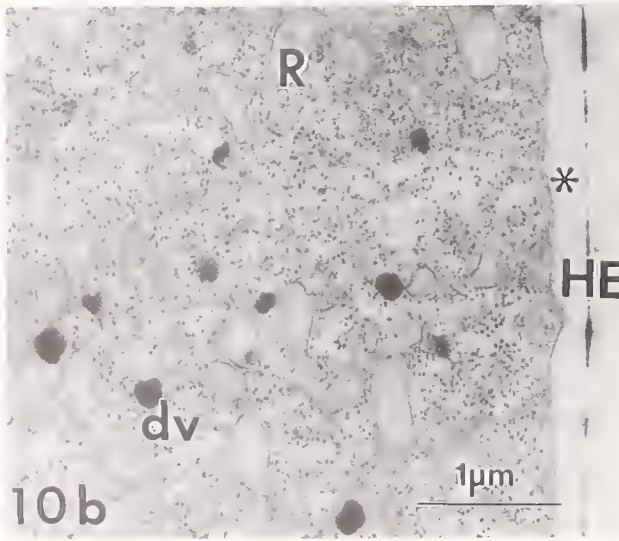
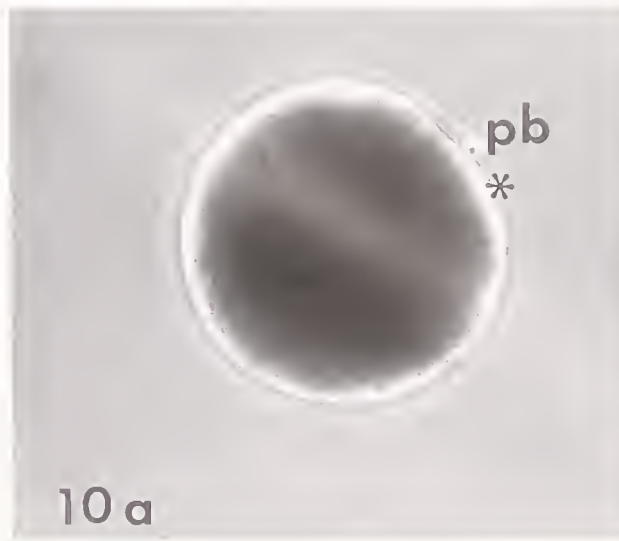
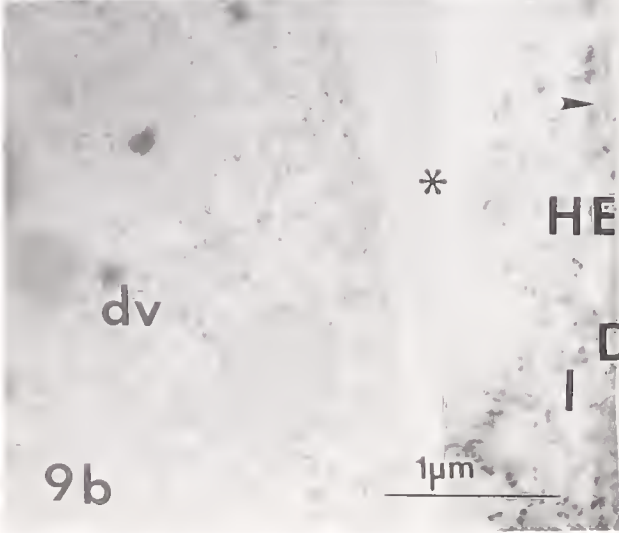
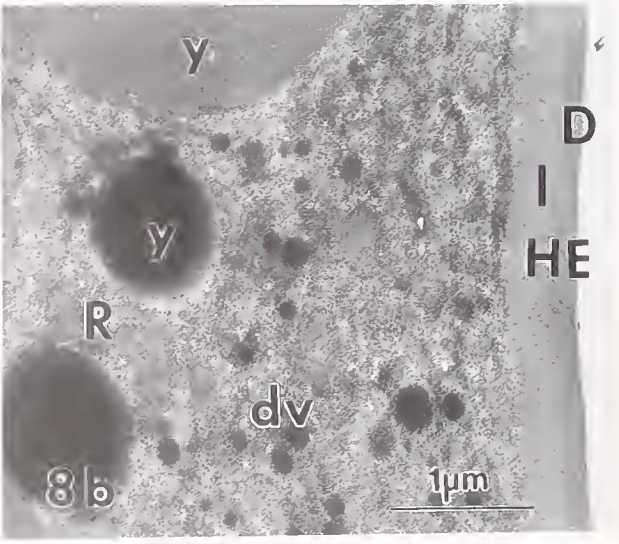
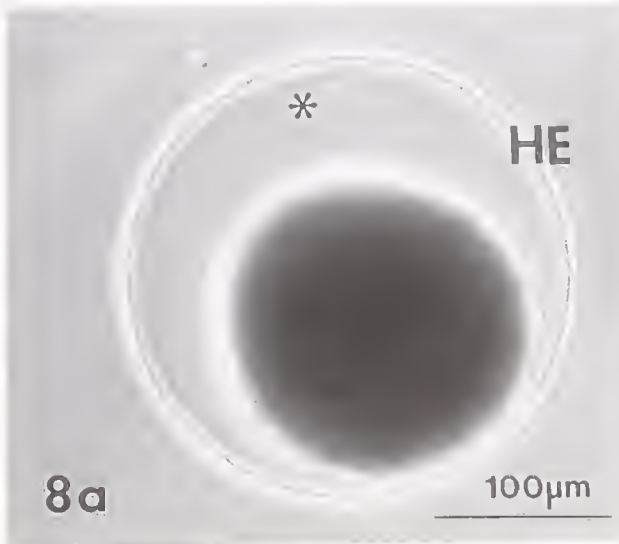
Eggs treated with tunicamycin and examined with TEM had a bilayer HE as in control samples, but the two layers had electron-translucent areas, or "holes," between the thin electron-dense outer layer and the flocculent inner layer (Fig. 2b). This condition resembled normal eggs during early elevation (*i.e.*, 60 min postspawn), suggesting a delay in HE formation (see Pillai and Clark, 1988, for comparison). Incorporation of

exocytosed ring vesicles into the HE was incomplete, and the ring elements remained conspicuous in the perivitelline space. The ER cisternae and the mitochondria were less conspicuous in the peripheral cortex (Fig. 2b) compared to control eggs. In TEM preparations of eggs treated with polyoxin D, the HE was abnormal and collapsed to the oolemma surface (Fig. 3b). The flocculent inner layer was interrupted by many large electron-translucent areas (holes). The outer layer and inner layer had a series of fine electron-translucent spaces separating the layers. The cortex of eggs treated in polyoxin D, unlike that of control eggs, was filled with prominent yolk platelets. The peripheral relocation of the yolk appeared to compress the ER and mitochondria in the egg cortex. Small electron-dense vesicles were not seen in the peripheral cortex. In TEM micrographs, eggs treated with nikkomycin Z lost the HE during processing (Fig. 4b), indicating an extremely fragile HE. Numerous vesicular structures were adjacent to the egg surface. The cortical region resembled that of an egg during early HE formation and elevation, with many small electron-dense vesicles and large vesicles containing a few ring elements.

Chitin hydrolytic enzymes inhibit normal envelope assembly

Sicyonia ingentis eggs were treated with the chitin hydrolytic enzymes (chitinase and *N*-acetylglucosaminidase) at 10 min postspawn and observed until 90 min postspawn. Chitinase-treated eggs formed HEs that initially elevated farther from the eggs than in the controls, then collapsed (Fig. 5a). Hatching envelopes were not apparent in *N*-acetylglucosaminidase-treated eggs in three out of three trials (Fig. 6a). When both *N*-acetylglucosaminidase and chitinase were added to the spawning media, again no HEs were discernible around the eggs (Fig. 7a),

Figures 5–7. Eggs of *Sicyonia ingentis* treated with chitinolytic enzymes do not elevate a normal hatching envelope (HE). (5a) Eggs treated with chitinase elevated HEs, but the envelopes appeared thinner than the control envelopes and usually collapsed to the oolemma surface. Bar equals 100 μm . (5b) The HE of eggs treated with chitinase appeared to have formed with the two layers, but the inner layer was not as thick as in control eggs. The dense layer (D) was separated from the inner layer (I) by small holes (arrowhead). The perivitelline space (*) contained finer material than seen in the control eggs. Endoplasmic reticulum cisternae and dense vesicles filled the egg cortex, but no packed ring vesicles were seen near the cortex. Bar equals 1 μm . (6a) Eggs treated with *N*-acetylglucosaminidase did not have elevated HEs. (6b) *N*-acetylglucosaminidase-treated eggs did not form normal HEs. Dense vesicles were seen in the cortex, and the packed ring vesicles were present, but the integrity of the cortical region was disrupted. Bar equals 1 μm . (7a) Eggs treated with both chitinase and *N*-acetylglucosaminidase did not elevate normal HEs. The eggs appeared swollen with a highly refractive cortex. (7b) Eggs treated with both chitinase and *N*-acetylglucosaminidase did not have HEs when viewed with TEM. The exterior of the oolemma resembled the surface of an egg in early HE formation. The large ring vesicles had only a few ring elements in them. Again, the structural integrity of the cortex appeared disrupted. Bar equals 1 μm . HE, hatching envelope; D, dense layer; I, inner layer; *, perivitelline space; R, ring vesicles; dv, dense vesicles; cis, endoplasmic reticulum cisternae; y, yolk platelets.



and the egg appearance resembled that of a newly spawned egg (see Pillai *et al.*, 1988). These eggs often appeared swollen, with a highly refractive cortex. Eggs in *N*-acetylglucosaminidase alone or both chitinase and *N*-acetylglucosaminidase were very fragile and broke easily when pipetted or swirled vigorously in the spawning dish.

With TEM, the HEs of the chitinase-treated eggs were bilayered, similar in appearance to control envelopes (Fig. 5b). The ER cisternae were as numerous as in control eggs, but appeared more electron-translucent, suggesting lack of material content. Small dense vesicles were abundant, but the large packed ring vesicles were not seen. TEM of *N*-acetylglucosaminidase-treated eggs showed a thin, unorganized layer of material resembling the HE precursor (see Pillai and Clark, 1988) on the exterior of the egg membrane (Fig. 6b). The eggs lacked structural integrity, suggesting eventual cell death. Vesicles with ring structures were smaller than in the control eggs. The small dense vesicles were similar to those in control eggs. When viewed with electron microscopy, eggs treated with a combination of both enzymes had a thin, unorganized area visible external to the oolemma (Fig. 7b); this area resembled the HE precursor. Dense vesicles were abundant in the cortex, and the large vesicles contained only a few ring elements. The leached appearance again suggested extreme disruption of cell function leading to cell death.

Ion deficient seawaters affect normal envelope assembly

In the following experiments, eggs were spawned in artificial seawater, washed three times in the appropriate ion-deficient seawater at 10 min postspawn, and allowed to develop. In calcium-deficient seawater, the HE, as observed with phase microscopy, elevated farther from the egg surface than in controls (Fig. 8a). The egg was usually eccentric within the HE, and the HE would collapse when eggs were handled. Elevation of HE by eggs in magnesium-deficient seawater appeared relatively normal

(Fig. 9a), although the HE did not appear to elevate as far from the egg as in the controls. Granular material similar to that in control eggs was visible in the perivitelline space. No elevated HE were observed in eggs treated with seawater deficient in both calcium and magnesium, although a thin HE precursor could be seen covering the second polar body (Fig. 10a). The eggs were very fragile, breaking easily with handling.

Examination of eggs from calcium-deficient seawater with electron microscopy showed an HE with the bilayer morphology, but the envelopes had collapsed to the oolemma (Fig. 8b), again suggesting lack of tanning of the envelope. Small dense vesicles were abundant in the cortex of the egg, but the mitochondria and cisternae were misshapen by yolk platelets. Eggs in magnesium-deficient seawater showed HEs with a thin bilayered construction (Fig. 9b). However, the HE had large electron-translucent areas ("holes") between the two layers and within the inner layer. The ring elements did not appear to be incorporated into the HE. The ER cisternae were prominent in the cortex. Ninety min postspawn eggs treated with calcium- and magnesium-deficient seawater showed HEs that were morphologically similar to HEs during early formation (Fig. 10b) (see Pillai and Clark, 1988, for comparison). The ring vesicles were not as packed as in the controls, and the cortex appeared similar to that of a normal egg during the period immediately before normal HE elevation and assembly.

Shrimp-isolated HEs label with β -glucoside specific lectins

Envelopes isolated from eggs at 90 min postspawn were incubated with fluorescently labeled lectins to detect the presence of carbohydrates: Concanavalin A (Con A) for mannose and *N*-acetylglucosamine, wheat germ agglutinin (WGA) for *N*-acetylglucosamine, *Lens culinaris* agglutinin (LCA) for mannose, and *Bandeirea simplicifolia* (BS-II) for *N*-acetylglucosamine. Untreated iso-

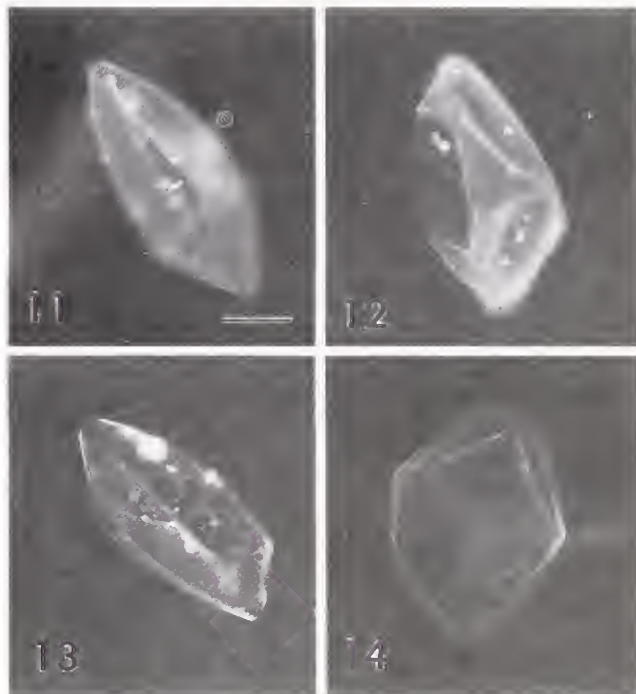
Figures 8–10. Eggs incubated in ion-deficient seawater do not elevate a normal hatching envelope (HE). (8a) Eggs treated with calcium-deficient seawater had an HE that appeared to elevate farther from the egg than in the controls. Bar equals 100 μ m. (8b) Eggs treated in calcium-deficient seawater had an HE that was collapsed to the oolemma, although a bilayered structure could be seen. Yolk and lipid droplets were close to the oolemma. Bar equals 1 μ m. (9a) In magnesium-deficient seawater, the HE did not elevate as far from the egg as in the controls. (9b) The HE in magnesium-deficient seawater was filled with electron-translucent areas, or "holes," between the two layers of the HE (arrowhead). The inner layer (I) was not as well organized as in the control or in calcium-free seawater. The micrograph is in two sections to allow a greater view of the cortex of the egg. Bar equals 1 μ m. (10a) In calcium- and magnesium-deficient seawater, the HE did not elevate from the egg surface although a thin HE precursor could be seen covering the second polar body (pb). (10b) In calcium- and magnesium-deficient seawater, a normal HE was not formed. On the exterior of the oolemma was a thin layer, probably the HE precursor (HE_p), but the dense layer and inner layer had not formed. Bar equals 1 μ m. HE, hatching envelope; D, dense layer; I, inner layer; *, perivitelline space; R, ring vesicles; dv, dense vesicles; cis, endoplasmic reticulum cisternae; y, yolk platelets.

lates showed no fluorescence (not shown). LCA and ConA strongly labeled the isolated HEs (Figs. 11 and 12). WGA (Fig. 13) and BS-II (Fig. 14) showed much weaker staining, and BS-II showed only a slight reaction with the isolated envelopes.

Discussion

The focus of this study is on the formation and elevation of the shrimp HE after exposure of the eggs to chitin hydrolytic enzymes or chitin synthesis inhibitors. However, the potential role of the cortex in the synthesis of any chitin-type carbohydrate cannot be ignored. Thus, the morphology of the cortex is an important aspect of HE formation. The cortical region of the penaeid shrimp egg at 90 min postspawn has the classic features of a biologically active cell—abundant mitochondria, endoplasmic reticulum, and cisternae. At least two types of vesicles with morphologically different characteristics are present, suggesting additional synthetic activity. Observations in this laboratory have shown that the HE continues to have material added to it until the next envelope is constructed at 90–110 min postspawn. Consequently, biochemical changes such as cross-linking (tanning or hardening) of the HE and incorporation of new material into the HE may continue until much later than originally expected and ultimately result in the appearance of chitin-like components in the HE. The three experimental approaches used in this study, *i.e.*, inhibitory, hydrolytic, and ionic treatments, resulted in visible changes in the morphology and assembly of the HE, as well as in the morphology of the cortex. These results were dramatically different from the normal sequence of events described in earlier investigations (Pillai and Clark, 1988, 1990; Lynn *et al.*, 1991, for review). Interestingly, the treatments often result in eggs that appear to have been arrested in earlier stages of development.

When eggs are treated with chitin synthase inhibitors, HE elevation occurs, but the structure of the HE and the morphology of the cortical region of the egg are affected by the synthase inhibition. The collapse or loss of the HE during handling suggests extreme fragility or susceptibility to chemical stress. The severity of the morphological abnormalities differs with the actions of the chitin synthase inhibitors. This is not surprising because the inhibitors are known to react differently between the insects and other crustaceans (Cabib, 1987, 1991; Cohen, 1987). Tunicamycin is an antibiotic that blocks the formation of protein-carbohydrate linkages of the *N*-glycosidic type (Duskin and Mahoney, 1979). Polyoxin D is a specific inhibitor of fungal chitin synthase that is known to result in malformed endocuticular layers in the larvae of the butterfly, *Pieris brassicae*, a cabbage pest (Cohen, 1987).



Figures 11–14. Fluorescent micrographs show labeling of isolated hatching envelopes (HEs) from *Sicyonia ingentis* eggs at 90 min postspawn. Labeling as follows: (11) LCA lectin; (12) ConA lectin; (13) WGA lectin; (14) BS-II lectin. Note the relative decrease in labeling for HEs treated with WGA and BS-II. Untreated control HEs showed no background fluorescence. Bar equals 50 μ m.

Nikkomycin Z is a competitive inhibitor of chitin synthase in fungal cell walls (Cabib, 1987). Although these antibiotics are reported to inhibit chitin synthetic pathways, the possibility of action on other related or unrelated metabolic pathways in decapod development cannot be dismissed.

Treatment of the eggs with two enzymes that are considered to be part of the chitinolytic pathway results, again, in abnormal elevation and assembly of the HE. With chitinase treatment an HE is elevated and appears somewhat normal in structure, but the collapse of the HE suggests abnormal hardening (*i.e.*, cross-linking) of the envelope. Although preliminary assays indicated some proteolytic activity in the chitinase enzyme, proteolytic inhibitors could not be simultaneously used because of their deleterious effect on HE elevation and morphology. In addition, the chitinase results indicate relatively subtle changes in HE formation in contrast to what occurs in proteolytic treatments of the HE. Thus while proteolytic effects cannot be ruled out, the results of the chitinase treatment are not consistent with such effects. Eggs treated with *N*-acetylglucosaminidase, the chitinolytic enzyme, appeared not to elevate an HE. The

chitinolytic enzymes do not appear to degrade the HE from the outside, as might be expected; instead, they seem to affect the assembly *per se*. Since the pH optima for chitinase and *N*-acetylglucosaminidase are 6 and 4, respectively (Muzzarelli, 1977), hydrolytic activity is unlikely to occur at the pH of seawater (pH 8.0). Thus, these enzymes are probably binding to substrate and effectively blocking further polymerization. The leached appearance of the cortex of eggs treated with *N*-acetylglucosaminidase suggests that one mode of action may be the disruption of the cortical architecture. Whether this disruption interferes directly with the release of a necessary substrate or enzyme or indirectly through cell death is unclear. However, the assembly of the HE in the presence of chitinase alone suggests that the *N*-acetylglucosaminidase is necessary for the lack of HE formation, much as the combination of enzymes is necessary for complete degradation of chitin.

Changes in ionic composition of the seawater after initial egg-sperm interaction has occurred also affect the assembly of the HE. Deficiency of Mg^{+2} and Ca^{+2} ions results in eggs with abnormal HE formation, cortical composition, or both (Clark and Lynn, 1977; Pillai and Clark, 1987). In the eggs treated with ion-deficient seawater, the holes between the two HE layers and within the inner layer suggest lack of incorporation of materials into the HE. In particular, the packed ring vesicles seem to be affected by these ionic deficiencies, as treated eggs were lacking the large number of packed ring vesicles found in the control eggs. The morphology of ion-depleted eggs suggests an interruption or delay in HE formation. As suggested for early egg activation (Clark and Lynn, 1977; Pillai and Clark, 1987), Mg^{2+} appears to be more important than Ca^{2+} for normal envelope assembly. The presence of at least one of these ions is necessary for normal HE assembly.

The lectin study showed some differences from results reported by Pillai and Clark (1990). However, the difference in the time points of the samples may account for some of the discrepancies. The results suggest that although glucose and mannose residues are present in the HE, labeling for *N*-acetylglucosamine oligomers by BS-II and WGA suggested either less accessibility or less abundance compared to the results of Pillai and Clark (1990).

The results of this study, together with previous demonstration of an oxidase (Glas *et al.*, 1995), offer argument for the presence of a chitin-like, or similarly linked, carbohydrate component in the HE of penaeid shrimp. The morphology of the penaeid egg during early development has all the characteristics of a biochemically active cell. The construction of the HE and the extra-embryonic envelopes that are formed later is a dynamic

function of this cell. Further exploration of the enzymes and resulting events during this period could offer new insights into the mechanics of embryo protection and activity during early development.

Acknowledgments

The authors express their appreciation to Dr. Wallis Clark and his students and colleagues for lab space at Bodega Marine Lab (UC-Davis) and for their patience and assistance in the procurement of *S. ingentis*. We also thank Eleanor Uhlinger for her assistance in obtaining reference materials and Ron Bouchard and Cindy Henk of LSU Life Sciences Microscopy Facility for assistance with the microscopy and photography. Rachel Arnold, Kathryn Kreimborg, and Shayd Coots were invaluable in their assistance with the laboratory work.

This work was supported in part by Louisiana Sea Grant #NA89-AA-D-SG226 project #R/SA-I to JWL and JDG and Sigma Xi Grant-in-Aid of Research to PSG.

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Metabolism of Gemmules From the Freshwater Sponge *Eunapius fragilis* During Diapause and Post-Diapause States

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Abstract. Post-diapause gemmules of the freshwater sponge *Eunapius fragilis* remained quiescent when maintained at 5°C. Germination occurred within 48 to 72 h following warming to 20°–23°C, culminating with the emergence of a new sponge from the collagenous capsule. Both heat dissipation and oxygen consumption climbed steadily during germination and eventually reached 600% of the starting values. By comparison, energy flow was much lower over the same period of time in diapausing gemmules, clearly demonstrating metabolic depression during diapause. The calorimetric:respirometric (CR) ratio increased significantly from –354 kJ/mol O₂ to –541 kJ/mol O₂ between hours 3.5 and 56.5 of germination, with an average value across this period of about –495 kJ/mol O₂. The low CR ratio at hour 12.5 (-374 ± 21 ; ± 1 SE, $n = 3$) was statistically below the oxycaloric equivalent, which suggests that gemmules may have experienced hypoxia during the more than 3 months of storage at 5°C prior to experiments. The increase in metabolism during germination could be blocked by perfusing the gemmules with nitrogen-saturated medium (nominally oxygen free). Developing gemmules were able to survive oxygen limitation for several hours at least; during that time energy flow was depressed to 6% of normoxic values. During germination, the range of values was 3.5 to 4.0 nmol/mg protein for ATP, 0.2 to 0.4 nmol/mg protein for ADP, and 0.5 to 0.8 nmol/mg protein for AMP. Because ATP was high even before gemmules were warmed to room tempera-

ture, it is unlikely that levels were severely compromised during the diapause condition.

Introduction

Gemmules of the sponge *Eunapius fragilis* are asexually produced reproductive bodies composed of undifferentiated cells surrounded by a complex collagenous capsule. Such bodies serve as the overwintering stage in the life cycle of this sponge. The possession of resting stages in the life cycles of invertebrates is common for organisms inhabiting inconsistent or ephemeral environments and is widespread phylogenetically (Hand, 1991). Newly formed gemmules are in an obligate state of developmental (and potentially metabolic) arrest that is termed diapause. Vernalization in the cold for 2–3 months releases the gemmules from diapause and allows them to resume development when warmed to 20–23°C.

The molecular mechanisms of this diapause breakage are unexplained. *E. fragilis* is well-suited for use in investigating these mechanisms because diapause in its gemmules is easily manipulated and is quite distinct from quiescence (Fell, 1987), a metabolically arrested condition promoted directly by environmental insult. In other sponges, these two states are apparently intertwined (e.g., *Spongilla lacustris*, Simpson and Rodan, 1976), and it can be quite difficult to distinguish whether or not gemmules are in diapause or whether the state has been broken. Other species apparently do not enter diapause at all and exhibit only quiescence (*Ephydatia fluviatilis*, Rasmont, 1963, 1965).

Received 10 October 1995; accepted 27 September 1996.

The ecological importance of dormant states has been emphasized by a number of authors (*e.g.*, Clutter, 1978; Crowe and Clegg, 1973; Danks, 1987; Elgmork, 1980; Hand, 1991; Hand and Hardewig, 1996; Hairston, 1987; King, 1980; Lees, 1955; Pourriot and Snell, 1983; Tauber *et al.*, 1986). A recent example of the ecological relevance of resting stages comes from observations on copepods and rotifers, where sediment cores from marine environments have uncovered viable embryos that have apparently withstood severe hypoxia or anoxia for 10–40 years; these forms are now thought to serve as “egg banks” for future generations (Marcus *et al.*, 1994). A report on diapausing eggs from freshwater copepods indicate that even longer survival times are possible (Hairston *et al.*, 1995).

Without metabolic energy conservation, survivorship during dormancy would be greatly reduced. General principles governing metabolic arrest are beginning to emerge (for reviews see Guppy *et al.*, 1994; Hand, 1991, 1993a, b; Hand and Hardewig, 1996; Hochachka and Guppy, 1987; Storey and Storey, 1990). Recent physiological studies have investigated cues associated with the termination of diapause (Drinkwater and Clegg, 1991; Drinkwater and Crowe, 1987, 1991; Lavens and Sorgeloos, 1987; van der Linden *et al.*, 1988) and have documented the metabolic transitions associated with this state (Clegg *et al.*, 1996). In this study, we wanted to establish a quantitative metabolic framework from which to ultimately investigate molecular mechanisms of diapause breakage. We employed microcalorimetry (Gnaiger, 1983a; Hand and Gnaiger, 1988) to assess overall energy flow in diapausing and post-diapause gemmules. Oxygen consumption was measured both simultaneously and independently of the calorimetric measurements. Finally, we measured ATP, ADP, and AMP in post-diapause gemmules to gain insight into the adenylate status during development.

In the accompanying paper (Loomis *et al.*, 1996), we address changes in carbohydrate levels during germination and potential enzymatic mechanisms for modulating these changes. Results of these studies could have general implications for organisms or tissues in which acute modulation of development and energy metabolism are known to occur. From an ecological perspective, the ongoing work may eventually provide a better understanding of how environmental stimuli, ones promoting entry into and exit from dormancy, are linked to the requisite physiological responses of invertebrates.

Materials and Methods

Diapausing gemmules were collected from Stony Brook in South Hadley, Massachusetts, in late Septem-

ber of 1993 and maintained at 4°C until early October when calorimetry was performed (storage for 2 weeks at 4°C is insufficient time to break diapause). Post-diapause gemmules were collected from the same location in February of 1993 and maintained for an additional 3 months in the laboratory at 4°C prior to metabolic experiments. The hatching percentage of post-diapause gemmules was always greater than 95%.

Closed-system respirometry

Experiments were initiated by filtering vernalized gemmules from the 4°C storage water and placing them into 20°C stream water that was continuously bubbled with air. Respiratory measurements were made at the indicated time intervals by transferring samples of gemmules to a sealed respiration vessel containing air-saturated stream water. Partial pressure of oxygen was measured using a model 5300 Yellow Springs Instruments biological oxygen monitor. Gemmules were continuously stirred through the 15-min measurement interval. The rate of oxygen consumption was linear down to 40% saturation, and during these experiments the oxygen level never dropped below 60% saturation.

Flow-through calorimetry and respirometry

Heat dissipation and oxygen consumption of gemmules were measured simultaneously with an LKB 2277 thermal activity monitor coupled *via* stainless steel tubing to a twin-flow respirometer (Cyclobios, Innsbruck, Austria) (Gnaiger, 1983a). All experiments were performed at 21°C. At time zero, gemmules were filtered from the 4°C stream water, transferred to the calorimeter, and perfused at 15 ml/h with 21°C air-saturated stream water (<10 mOsm/kg H₂O; 0.22 μ m filtered). The partial pressure of oxygen was above 60% air saturation on average in the excurrent flow after passage by the gemmule sample. One data point was sampled every 60 s and recorded with Datgraf acquisition software (Cyclobios; Innsbruck, Austria). After each run, the gemmules were removed and dried at 60°C to constant weight. Baselines were measured before and after each run, without gemmules in the perfusion chamber. For the experiment involving a bout of oxygen limitation, post-diapause gemmules were given aerobic perfusion for 21 h, and then the perfusion was switched to nitrogen-saturated stream water for 7.5 h. After the bout of oxygen limitation, gemmules were returned to aerobic perfusion to initiate a recovery period. One advantage of flow-through calorimetry for such experiments is the ease and speed with which oxygen availability can be manipulated. Although considerable effort is devoted to main-

taining a nominally oxygen-free status in the flow-through system (for methodological precautions employed, see Gnaiger 1983a; Hand and Gnaiger, 1988; Hand, 1990), evidence indicates that a sealed ampoule system, while experimentally cumbersome in terms of reversibility, may be slightly more effective in providing an anoxic environment (Hand, 1995).

The influence of antibiotics (50 mg/l each of penicillin and streptomycin) on the heat dissipation was evaluated by exposing gemmules for 5 h to perfusion with nitrogen-saturated medium and then switching to the same nitrogen-saturated medium containing antibiotics. The slope of the μW -versus-time curve before the switch to antibiotics was compared to the slope 4 h after the switch. The slopes were not significantly different ($P = 0.343$, $n = 3$, paired, two-tailed t test). Similarly, the absolute μW values before and after the switch were not significantly different ($P = 0.258$, $n = 3$, paired, two-tailed t test). Thus, antibiotics were not included in the water used for calorimetry experiments.

Adenylate measurements

Post-diapause gemmules were incubated aerobically in stream water at 20°–23°C. At the indicated time points, triplicate samples were extracted with perchloric acid (Rees and Hand, 1991). Perchloric acid precipitates were resuspended in 0.5 *N* NaOH and assayed for total protein with a modified Lowry assay (Peterson, 1977). The neutralized supernatants were analyzed for adenylates by high-performance liquid chromatography using weak anion exchange as previously described (Rees and Hand, 1991). Briefly, the separation column of amino propyl silica (250 × 4.6 mm, 5 μ m particle size; Phenomenex, Torrance, CA) was eluted isocratically with a 2:1 mixture of acetonitrile and 60 mM potassium phosphate buffer (pH 6.5). The potassium phosphate replaced the ammonium bicarbonate used by Rees and Hand (1991) in the mobile phase, which avoided the changes in composition caused by slow outgassing of CO₂ from the mixture. Adenylates were monitored at 254 nm and identified by co-elution with bona fide standards.

Results

Heat dissipation and oxygen consumption of gemmules

A pronounced increase in heat dissipation of 5.8-fold is observed upon incubating post-diapause gemmules at 21°C (Fig. 1). In comparison, two distinct differences are seen with diapausing gemmules (Fig. 1). First, the absolute level of heat dissipation after 3.5 h of incubation at 21°C is 50% of that measured for the post-diapause gem-

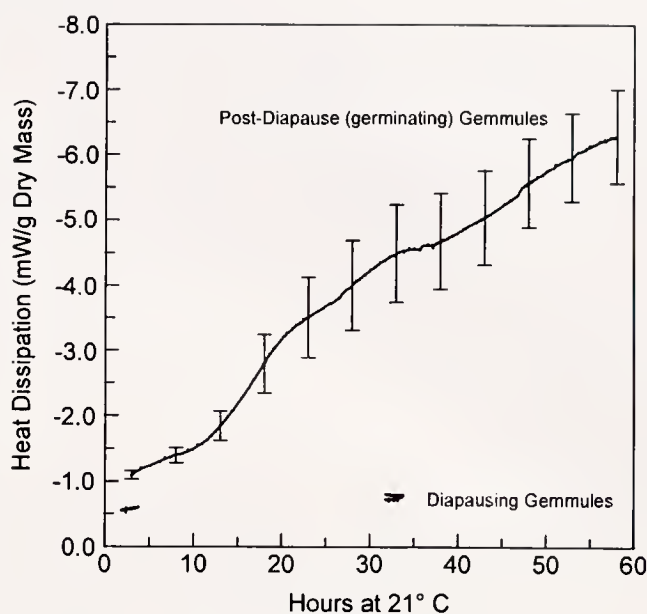


Figure 1. Calorimetric measurements of energy flow in germinating and diapausing gemmules of *Eunapius fragilis* at 21°C. Data for germinating gemmules are expressed as means of three independent experiments; bars represent one standard error of the mean at the time points indicated. Values for diapausing embryos are presented for two independent experiments at the time intervals shown.

mules at the same temperature. Thus, breakage of diapause apparently results in an increased heat flow. Second, the large increase in heat dissipation observed over time for the post-diapause condition is absent with the diapausing embryos. Even though the diapausing embryos are presented with optimal conditions for development and germination, they remain dormant (no morphological signs of germination were observed). These quantitative data illustrate the developmental and metabolic differences between these two states of diapause and post-diapause.

A similar overall trend is observed when respiration rate is plotted as a function of developmental time (Fig. 2). As seen in the inset to Figure 2, closed-system respirometry provides results comparable to those obtained with the open-flow system. Figure 3 depicts the changes in the CR ratio during development of post-diapause embryos. The value increases significantly ($P = 0.023$, unpaired, two-tailed t test) from -354 ± 46 kJ/mol (± 1 SE, $n = 3$) to -541 ± 36 kJ/mol (± 1 SE; $n = 4$) between hours 3.5 and 56.5 of germination, with an average value of about -495 kJ/mol across this period. For comparison, the oxycaloric equivalent for carbohydrate catabolism under aerobic conditions is -478 kJ/mol O₂ (Gnaiger, 1983b, c). The CR value of -374 ± 21 (± 1 SE;

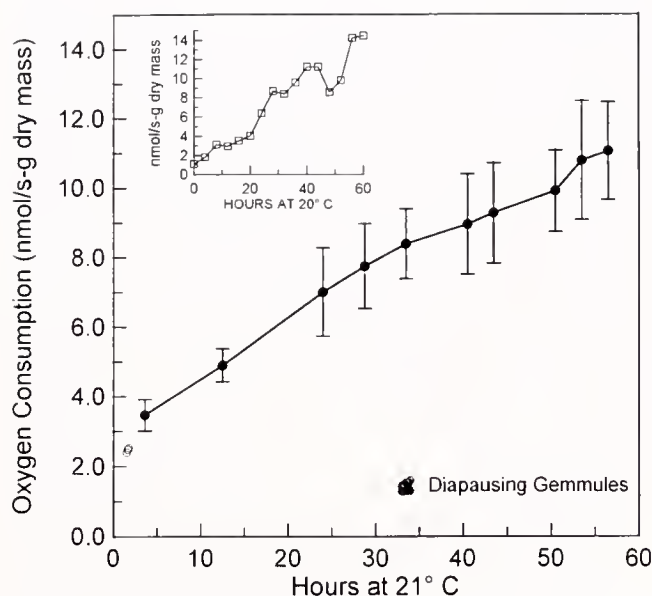


Figure 2. Oxygen consumption rates at 21°C, measured using the flow-through respiration system, for post-diapause and diapausing gemmules of *Eunapius fragilis* during germination. Values for post-diapause gemmules are means for 3-h intervals (except for a 1.25-h interval at the first time point), with the symbol placed at the mid-point of each time interval. Error bars represent ± 1 SE for three independent experiments, except at hours 50.5 and 56.5, where $n = 4$. Values for diapausing embryos are presented for two independent batches of embryos at the time intervals shown. Inset: For comparison, respiration rates measured using closed-system respirometry at 20°C are presented for post-diapause gemmules; $n = 1$.

$n = 3$) determined at hour 12.5 is statistically different from the oxycaloric equivalent ($t_1 = 4.95$). This lower C:R ratio near the beginning of the incubation could be attributed to gemmules that are undergoing recovery from hypoxia or anoxia. Because they were stored for months at 4°C without aeration, it is likely that these quiescent gemmules could have experienced oxygen limitation.

Tolerance of gemmules to oxygen limitation

Figure 4 illustrates the effect of interrupting the aerobic germination of post-diapause gemmules with a 7.5-h bout of oxygen limitation. Heat flow is depressed to 6% of the aerobic rate. Upon reperfusion with air-saturated medium (at hour 28), heat flow increases, and in due course, new sponge tissue emerges from the collagenous capsule. Emergence is delayed by an increment of time equal to the bout of nitrogen perfusion (data not shown). Thus, oxygen limitation appears to halt development in addition to depressing heat dissipation. These observations are similar to those reported for embryos of the

brine shrimp *Artemia franciscana* (Hand and Gnaiger, 1988; Hand, 1990). Even the biphasic profile seen with the gemmules during recovery (hours 28–30) is virtually identical to that observed with *A. franciscana* embryos.

Adenylate levels during germination

Considerable variance was noted in the ATP levels of gemmules during the early stages of the germination process, as manifested in the large error bars over the first 20 h (Fig. 5). Overall, however, the mean values are relatively consistent across the entire period. There is a small decrease in ADP at hour 42 (Fig. 5) that results in a significant change ($P = 0.008$) in the ATP:ADP ratio when compared to hour 0 (Table I). AMP levels fluctuate between 0.5 and 0.8 nmol/mg protein (Fig. 5). The calculated adenylate energy charge was statistically unchanged over the 54 h of aerobic incubation (Table I). Even at the hour 0 time point (*i.e.*, immediately before transferring the post-diapause gemmules to room temperature), the adenylate pool is already well charged. The

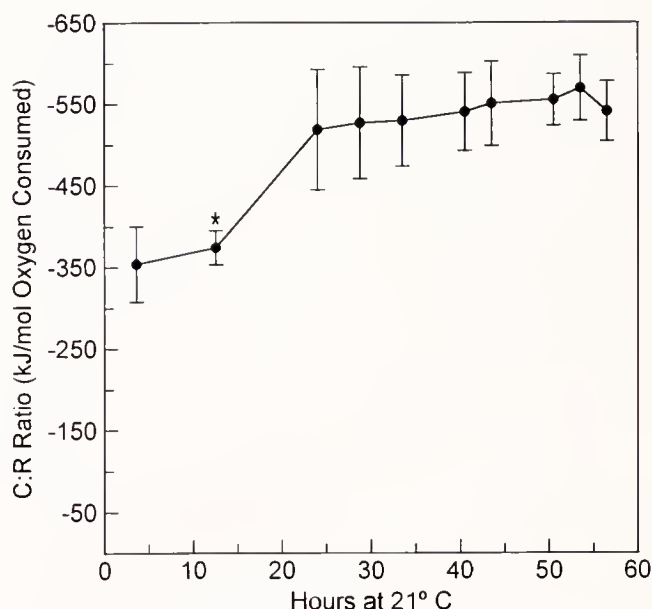


Figure 3. Calorimetric:respirometric (CR) ratios for post-diapause gemmules of *Eunapius fragilis* during germination at 21°C. Values are means for 3-h intervals (except for a 1.25-h interval at the first time point), with the symbol placed at the mid-point of each time interval. Error bars represent ± 1 SE for three independent experiments, except at hours 50.5 and 56.5, where $n = 4$. Analysis of variance shows a significant increase in CR ratio between the first and last time points ($P = 0.023$; unpaired, two-tailed t test). The asterisk denotes the only value significantly different ($t_1 = 4.95$) from the theoretical oxycaloric equivalent for carbohydrate (-478 kJ/mol O_2), based on comparison of the experimental means and associated variances to the parametric value.

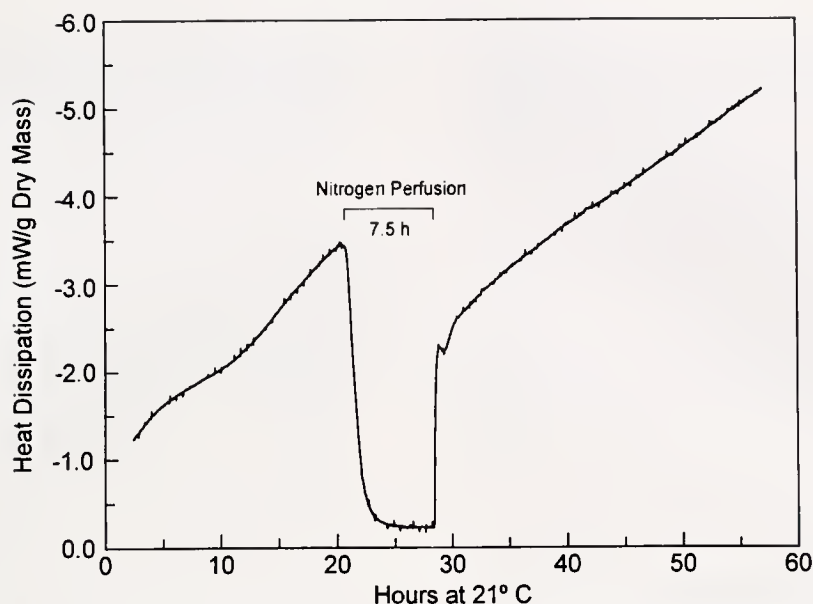


Figure 4. Interruption of heat dissipation during germination of *Eunapius fragilis* gemmules by perfusion with nitrogen-saturated medium (nominally oxygen free). The period of oxygen deprivation is indicated on the figure.

observation implies that adenylate status is probably not compromised by the diapause period prior to reinitiation of differentiation. Otherwise, one would expect to see a substantial rise in ATP levels during the early stages of germination.

Discussion

Energy flows in gemmules of the freshwater sponge *Eunapius fragilis* differ between the states of diapause

and post-diapause (germination). Diapausing gemmules exhibit an energy flow only 50% that of post-diapause gemmules at the beginning of germination, and this rate of heat flow is only 11% of that seen in post-diapausing gemmules at the point of cell emergence from the collagenous capsules. Thus, diapause is characterized by a substantial metabolic and developmental suppression. Nevertheless, there is still energy metabolism in this state of obligate (constitutive) dormancy when measured at 21°C. Presumably, the energy flow during diapause would be depressed further by the low temperatures naturally experienced during the overwintering state. Gas-

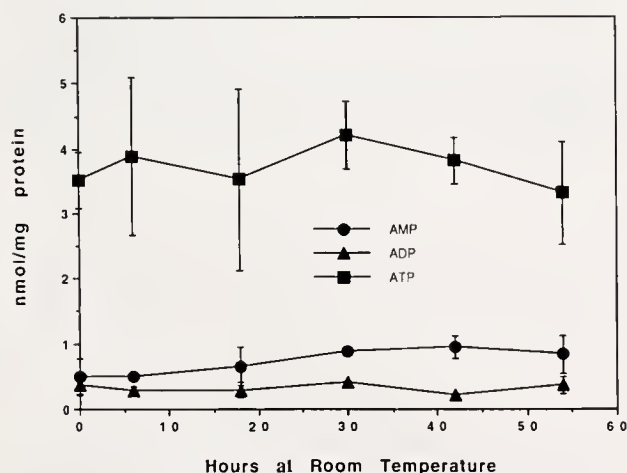


Figure 5. Chemical measurements of ATP, ADP, and AMP extracted from gemmules of *Eunapius fragilis* at various points during germination. Values are expressed as means $\pm$ 1 SE ($n = 3$).

Table I

ATP:ADP ratios and adenylate energy charge in gemmules of *Eunapius fragilis* during germination

Time at room temperature (h)	ATP:ADP ratio ^a	Energy charge ^a
0	6.91 $\pm$ 0.96	0.81 $\pm$ 0.02
6	15.12 $\pm$ 1.31	0.86 $\pm$ 0.01
18	12.15 $\pm$ 1.89	0.83 $\pm$ 0.01
30	10.28 $\pm$ 0.05	0.80 $\pm$ 0.01
42	17.55 $\pm$ 0.92*	0.79 $\pm$ 0.01
54	9.86 $\pm$ 2.57	0.77 $\pm$ 0.05

^a Values represent means $\pm$ 1 SE ($n = 3$).

* Significantly different from hour 0 value ($P = 0.008$), based on ANOVA with Bonferroni adjustments for multiple comparisons. No significant differences were found for the energy charge values.

trula-stage embryos of the brine shrimp *Artemia franciscana* exhibit an exponential decline in respiration rate as a function of time in diapause; after 5 days of diapause, respiration reached "barely detectable" levels (Clegg *et al.*, 1996). Considering that gemmules in this study had been in diapause for 2 weeks before the metabolic measurements, it seems that gemmules of *Eunapius fragilis* do not undergo as severe a metabolic depression as that reported for brine shrimp embryos.

Respiration rates of diapausing and post-diapause gemmules were compared by Rasmont (1962), who found that oxygen consumption increased threefold upon breakage of diapause (promoted by cold treatment of gemmules). These studies were conducted with gemmules of *Ephydatia muelleri*, which apparently do not exhibit as distinct a diapause state as do gemmules of *Eunapius fragilis*. The current evidence suggests that there is a metabolic activation that coincides with the resumption of development after diapause breakage and subsequent exposure to elevated temperatures.

Over the entire period of germination, the CR ratio for gemmules of *Eunapius fragilis* averaged -495 kJ/mol O_2 , a value indistinguishable from the oxycaloric equivalent for aerobic metabolism of carbohydrate of -478 kJ/mol O_2 . As noted previously, however, the CR ratio was significantly lower ($-374 \pm 21 \pm 1 \text{ SE}$; $n = 3$) at hour 12.5 of the germination process (similarly low at hour 3.5), an observation that leads us to suspect that the gemmules were hypoxic prior to the aerobic incubation period of germination. Values significantly below the oxycaloric equivalent have been reported before for organisms recovering from anoxia. For the marine mussel *Mytilus edulis*, values as low as -210 kJ/mol O_2 have been observed during the first hour of recovery from anoxia (Shick *et al.*, 1986; Shick *et al.*, 1988). A small portion of the excess oxygen consumed (relative to heat liberated) can be attributed to reoxygenation of tissues, but the predominant cause of the low CR ratios is apparently the occurrence of anabolic, heat-conserving metabolism (Shick *et al.*, 1986; Shick *et al.*, 1988). The theoretical oxycaloric equivalent for gluconeogenic succinate clearance is estimated to be about -200 kJ/mol O_2 , and the oxycaloric equivalent for the partial oxidation of succinate to malate or aspartate is projected to be quite low as well (reviewed by Shick *et al.*, 1988). Further support for this argument comes from the calculated CR ratio soon (2.5 to 3.5 h) after the short anoxic bout in Figure 4. The value was a low -363 kJ/mol O_2 , compared to -438 kJ/mol O_2 immediately before the anoxic bout (hour 19.5–20.5). By 3.5–6.5 h post-anoxia, the CR ratio had risen to -457 kJ/mol O_2 and to -527 kJ/mol O_2 by 10.5–13.5 h post-anoxia.

Adenylate pools appear to be well charged in post-diapause gemmules even before warming to room temperature (hour 0). Preliminary data (not shown) suggest that ATP levels are also quite high in diapausing gemmules, and consequently, severe depletion of ATP is unlikely in either of these two metabolic states. Data are currently not available for anoxic gemmules, but our prediction is that ATP levels could be compromised under anoxia, similar to the situation observed for *Artemia* embryos, where ATP drops rapidly during oxygen limitation (Anchordoguy and Hand, 1994, 1995; Carpenter and Hand, 1986; Hand and Gnaiger, 1988; Rees *et al.*, 1989; Stocco *et al.*, 1972).

Post-diapause gemmules are tolerant of severe hypoxia or anoxia, at least for a period of 7.5 h. During nitrogen perfusion (nominally oxygen free), heat dissipation is reduced to 6% of the aerobic value. In comparison, after 50 h of oxygen deprivation, heat dissipation from post-diapause embryos of *Artemia franciscana* drops at least an order of magnitude and is still declining at that point (Hand, 1990, 1995; compare with Hontoria *et al.*, 1993). It is possible that such low values of heat dissipation in *Artemia* embryos reflect an ametabolic state, as defined by the absence of processes associated with ATP turnover. Upon return of gemmules to aerobic perfusion, heat dissipation climbs rapidly, and gemmules resume development and emerge. Exploration of biochemical and metabolic features underlying this tolerance to oxygen limitation and the capacity for long-term survivorship under anoxia is biologically relevant because the natural habitat of these gemmules during winter can include burial in pond sediments of low oxygen tension (S. H. Loomis and P. E. Fell, unpubl. observations).

The results from this study, in conjunction with those of the companion paper, provide a quantitative framework from which to explore molecular bases for diapause. The data represent the first characterization of the metabolic transitions in sponge gemmules that are capable of entering and exiting diapause.

Acknowledgments

This work was supported by NSF grants DCB-9018579 and IBN-9306652 to SCH.

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Carbohydrate Mobilization During Germination of Post-Diapausing Gemmules of the Freshwater Sponge *Eunapius fragilis*

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Abstract. Post-diapausing gemmules of the freshwater sponge *Eunapius fragilis* were found to contain sorbitol and glycogen as their primary carbohydrates. The sorbitol probably acts to increase the tolerance of the gemmules to freezing and desiccation. During germination, average sorbitol levels—measured as micromoles of sorbitol per gram of fresh weight of gemmule tissue ($\mu\text{mol/gfw}$)—declined from a control value of $36 \mu\text{mol/gfw}$ to about $4 \mu\text{mol/gfw}$. Concomitantly, average glycogen levels increased from a control value of $29 \mu\text{mol/gfw}$ to a steady-state level of $62 \mu\text{mol/gfw}$. It is probable that glycogen is being synthesized at the expense of sorbitol. The breakdown of sorbitol was associated with an increase in the activity of sorbitol dehydrogenase from undetectable levels in dormant gemmules to a maximum of $0.2 \mu\text{mol/min} \cdot \text{mg}$ protein after 30 h of exposure to 20°C . Aldose reductase activity remained constant throughout germination. These data support the hypothesis that the decrease in sorbitol levels is the result of an increase in the rate of catabolism by sorbitol dehydrogenase. The total activity of glycogen synthase did not change during germination; however, the activity of glucose-6-phosphate-dependent glycogen synthase was about 18 times greater than the activity of glucose-6-phosphate-independent glycogen synthase. Total glycogen phosphorylase activity increased from about $1.6 \text{ nmol/min} \cdot \text{mg}$ protein to $3.6 \text{ nmol/min} \cdot \text{mg}$ protein during germination. At the same time, however, the percentage of glycogen phosphorylase *a* decreased from almost 100% to about 84%.

This decrease would attenuate the apparent increase in activity. cAMP levels remained constant throughout germination. The observed changes in the level of glycogen in the gemmules are not simply due to changes in the activity of either glycogen phosphorylase or glycogen synthase.

Introduction

The life cycle of many freshwater sponges includes the formation of structures called gemmules. These consist of a mass of undifferentiated cells enclosed by a collagenous capsule. Gemmules are resistant to many environmental perturbations and are often produced as the sponge enters a period of environmental stress. For example, in southern New England the freshwater sponge *Eunapius fragilis* over-winters in the form of gemmules. After the sponge produces gemmules in the early fall, the adult tissue dies, leaving the gemmules attached to the substratum. In spring, the gemmules germinate to form the adult sponge again (Fell, 1987). In contrast, in temporary swamps in the southeastern United States the same species produces gemmules as the swamp dries in the summer. In this case the gemmules are exposed to hot, dry conditions and germinate when the swamps form again (Poirrier, 1969).

The physiological state of the gemmules from *Eunapius* changes with time and can be divided into three distinct stages: (1) diapause, (2) quiescence, and (3) germination (Fell, 1987). As gemmules are formed by *Eunapius fragilis*, they are initially in diapause, a state of dormancy controlled by some as yet unknown endoge-

nous factor or factors. In this state, the gemmules will not germinate even if exposed to favorable conditions. For example, gemmules that are collected in New England just after formation will not germinate when they are warmed to 20°C, even if kept at that temperature for up to 4 months. Quiescence occurs in gemmules collected at least 3 to 4 months after formation. In this case, the metabolism and development of gemmules is simply being depressed by low temperature, and they will readily germinate when warmed to 20°C. Similarly, in the laboratory, diapause can be broken by exposing the gemmules to cold (5°C) for 4 to 5 months or by exposing them to 5°C for 4 to 6 weeks followed by desiccation for 7 days (Fell, 1987). The breaking of diapause represents a basic shift from endogenous control of dormancy to environmental control of quiescence.

Germination of gemmules represents the period of development leading from post-diapause quiescence to the differentiated adult sponge. In *Eunapius*, emergence of the adult sponge tissue from the gemmule occurs 42 to 50 h after exposure to 20°C. Very little is known of the biochemical changes that occur during germination. Almost all of the information about these changes is based on nonquantitative histochemical methods and is limited to compounds easily stained in thin section (Simpson, 1984). The purpose of this paper, therefore, is to identify the major carbohydrates present in the post-diapausing gemmules of *Eunapius fragilis* and to examine changes in the levels of these compounds during germination.

Materials and Methods

Gemmules

Gemmules used for the measurement of possible energy sources during germination were collected from Gallop Pond in North Stonington, Connecticut, in late October of 1991. Gemmules used in the enzyme studies were collected from Stony Brook in South Hadley, Massachusetts, during early February of 1992. All gemmules were kept at 5°C until June to ensure that diapause was broken.

Germination experiments

Samples of gemmules ranging in size from 100 to 300 mg wet weight were placed in individual test tubes containing 10 ml of filtered stream water at 5°C. At the initiation of the experiment, the cold stream water was decanted from all of the gemmule samples and was replaced by filtered stream water at 20°C. All samples were well aerated and maintained at 20–23°C. Periodically, the stream water was decanted from three of the gem-

mule samples, which were then homogenized according to the protocol for the particular experiment (as outlined below).

Extraction of carbohydrates

Glycogen. Glycogen was extracted from the gemmules with cold 80% ethanol. Gemmules (300 mg) were ground under liquid nitrogen in a mortar and pestle. The powder was transferred to 50-ml centrifuge tubes and the liquid nitrogen was allowed to evaporate. Five milliliters of 80% ethanol was added to the centrifuge tubes containing the powdered gemmules and was mixed thoroughly on a vortex mixer. The ethanol solution was placed at 4°C overnight to allow all of the glycogen to precipitate and was then centrifuged at $10,000 \times g$ for 20 min. The supernatant was retained for ethanol-soluble carbohydrate measurement and the pellet was washed twice more with 2 ml of cold 80% ethanol. The washings were combined with the original supernatant. The final pellet was dissolved in 5 ml of 30% KOH and heated to 100°C in a boiling water bath. After the sample was cooled to room temperature, 7.5 ml of 80% ethanol was added, and the glycogen was precipitated at 4°C overnight. Glycogen was collected by centrifuging the sample at $10,000 \times g$ for 20 min and the resulting pellet was washed twice more with cold 80% ethanol. The pellet was dried under nitrogen, then dissolved in 5 ml distilled water; the amount of glycogen in the solution was measured using the anthrone method of Umbreit *et al.* (1972). The amount of glycogen measured using this method was not significantly different from that measured by the amyloglucosidase-based assay.

Identification of ethanol-soluble carbohydrates

High-performance liquid chromatography (HPLC). The combined supernatant from the ethanol extraction was dried under nitrogen, redissolved in either H₂O (for HPLC) or D₂O (for NMR) and filtered through a 0.45- μ m filter prior to further analysis. Initial identification of carbohydrates in these samples was determined by HPLC using a Waters Radial-Pak silica cartridge modified with tetraethylenepentamine according to the methods of Hendrix *et al.* (1981). Carbohydrates were detected using an LDC/Milton Roy RefractoMonitor III.

Nuclear magnetic resonance (NMR). The ethanol-soluble carbohydrates were further characterized by ¹H- and ¹³C-NMR on a Bruker AC250 NMR. ¹H- and ¹³C-NMR spectra were measured at 250.1 and 62.9 MHz and chemical shift values are reported relative to the internal standard, sodium, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

Sorbitol. Sorbitol in the ethanol-soluble fraction was

measured enzymatically using the method of Bergmeyer *et al.* (1974). The incubation solution contained 1 unit of sorbitol dehydrogenase and 1.2 mM NADH in 200 mM sodium phosphate buffer, pH 9.5, in a total volume of 2.0 ml. The amount of sorbitol was measured by incubating aliquots (50 μ l) of standards and samples in the incubation medium for 30 min and measuring the change in absorbance at 340 nm.

Enzyme assays

Sorbitol dehydrogenase. Sorbitol dehydrogenase activity was measured at 25°C in extracts of gemmules according to a modification of the method of Wolff (1955). Gemmules (100 mg) were homogenized in a Tenbroek homogenizer in 300 μ l of grinding buffer (20 mM Tris-HCl, pH 7.32). The homogenate was centrifuged at $10,000 \times g$ for 10 min and an aliquot (100 μ l) of the supernatant was placed in 1.7 ml of assay buffer, resulting in a final concentration of 300 μ M NAD in 100 mM Tris-HCl, pH 9.0. A baseline rate was recorded, and the assay was then initiated by the addition of 200 μ l of 1.5 M sorbitol (150 mM final concentration). Activity was followed by measuring the rate of change in the absorbance at 340 nm.

Aldose reductase. Aldose reductase activity in extracts of gemmules was measured at 25°C using a modification of the method of Velle (1975). Gemmules were homogenized as described in the methods for sorbitol dehydrogenase and an aliquot (100 μ l) of the protein solution was placed in 1.6 ml of an assay buffer, resulting in a final concentration of 330 μ M NADPH in 300 mM Tris-HCl, pH 7.5. A baseline rate was recorded, and the assay was initiated by the addition of 300 μ l of 100 mM glyceraldehyde (45 mM). Activity was followed by measuring the rate of change in the absorbance at 340 nm. Aldose reductase has a broad substrate specificity and will catalyze the reduction of a number of aldoses (Hayman and Kinoshita, 1965). Glyceraldehyde (which results in the synthesis of glycerol) was used as a substrate in these assays instead of glucose (which results in the synthesis of sorbitol) to obtain increased sensitivity (Hayman and Kinoshita, 1965).

Glycogen synthase. Glycogen synthase activity was measured at 25°C in extracts of gemmules according to a modification of the methods of Mendicino *et al.* (1975). Gemmules (300 mg) were homogenized in 0.9 ml of 50 mM Tris-HCl, pH 7.5, containing 0.3 M sucrose, 20 mM 2-mercaptoethanol, and 5 mM EDTA. The homogenate was centrifuged at $10,000 \times g$ for 10 min and an aliquot (100 μ l) of the supernatant was added to 0.21 ml of an assay buffer, resulting in a final concentration of 117 mM Tris-HCl, pH 7.5, 29 mM cysteine, and

100 mg/ml of glycogen. Glucose-6-phosphate-dependent activity was measured by the addition of glucose-6-phosphate to the assay solution to a final concentration of 11.8 mM. The assay was initiated by the addition of UDP-[14 C]-glucose to a final concentration of 4.3 mM (specific radioactivity of 50,000 cpm/ μ mole). The assay was terminated after 30 min by the addition of 1 ml of 10% trichloroacetic acid. The resulting solution was centrifuged at $10,000 \times g$ for 10 min. The supernatant was removed and added to 4 ml of 95% ethanol (-20°C) to precipitate the glycogen. The glycogen was pelleted by centrifuging at $10,000 \times g$ for 10 min. The supernatant was discarded and the glycogen pellet was dissolved in 1 ml distilled water. The glycogen was reprecipitated twice more by adding 4 ml of 95% ethanol (-20°C) to this solution, followed by centrifugation. The amount of ^{14}C incorporated into the glycogen from UDPG during the incubation period was measured in a liquid scintillation counter.

Glycogen phosphorylase. Glycogen phosphorylase activity was measured at 25°C in extracts from gemmules according to a modification of the method of Mendicino *et al.* (1975). Gemmules (100 mg) were homogenized in 300 μ l of 50 mM imidazole buffer (pH 7.0), containing 10 mM magnesium chloride, 2 mM EDTA, 0.01% bovine serum albumin, and 1% glycogen. The solution was centrifuged at $10,000 \times g$ for 10 min and the supernatant was retained for the assay. Total activity was measured in 1 ml of 50 mM imidazole, pH 7.0, containing 10 mM magnesium chloride, 2 mM EDTA, 0.01% bovine serum albumin, 1.1% glycogen, 70 mM potassium phosphate, 300 μ M NADP, 10 μ M glucose-1,6-diphosphate, 4 mM AMP, 1 unit phosphoglucosylase, and 1 unit glucose-6-phosphate dehydrogenase. When phosphorylase *b* was measured, the AMP was omitted. The assay was initiated by the addition of 100 μ l of the protein solution. Activity was determined by measuring the rate of change in the absorbance at 340 nm. Protein concentration in extracts used for all enzyme assays was determined according to the methods of Peterson (1977).

cAMP assay. Adenosine 3',5' cyclic monophosphate (cAMP) in extracts from gemmules of *Eunapius fragilis* was measured using an ^{125}I radioimmunoassay according to the methods outlined in the Rianen (Dupont Medical Products) Assay System. Samples were prepared by homogenizing 30 mg of gemmules in 0.5 ml of cold (4°C) 6% trichloroacetic acid. cAMP recovery during extraction was determined by adding about 0.1 pmol (4,000 CPM) of ^3H cAMP to the homogenate as an internal standard. The extracts were centrifuged at $2500 \times g$ for 15 min, and the supernatant was extracted four times with 2.5 ml of water-saturated ether. The aqueous layers were then dried under a stream of air at 70°C . The

resulting residue was dissolved in 0.5 ml of the radioimmunoassay assay buffer, and cAMP concentrations were measured in 100- μ l aliquots of this solution.

Statistical analysis. Statistical differences among treatments were assessed by analysis of variance. Values are expressed as means $\pm$ 1 SE.

Results

Ethanol-soluble carbohydrates

HPLC analysis of the ethanol-soluble fraction showed a single peak that co-eluted with sorbitol and remained single when spiked with authentic sorbitol (data not shown). Because it is difficult to exclude the presence of other six-carbon polyols solely on the retention times on

the HPLC column, we used ^1H - and ^{13}C -NMR, which together can distinguish the isomeric hexitols, to further analyze the ethanol-soluble fraction. The spectra for both ^1H - and ^{13}C -NMR were virtually identical to the spectra, obtained under identical acquisition conditions, for authentic sorbitol (Fig. 1). The chemical shifts for ^{13}C -NMR are shown in Table 1. Further, there were no additional peaks in the spectra, eliminating the possibility of other compounds being present. Based on the HPLC and NMR data, it is clear that sorbitol comprises the major ethanol-soluble carbohydrate in post-diapause gemmules.

Sorbitol levels determined by sorbitol dehydrogenase and expressed as micromoles per gram of fresh gemmule tissue ($\mu\text{mol/gfw}$) declined rapidly in germinating gem-

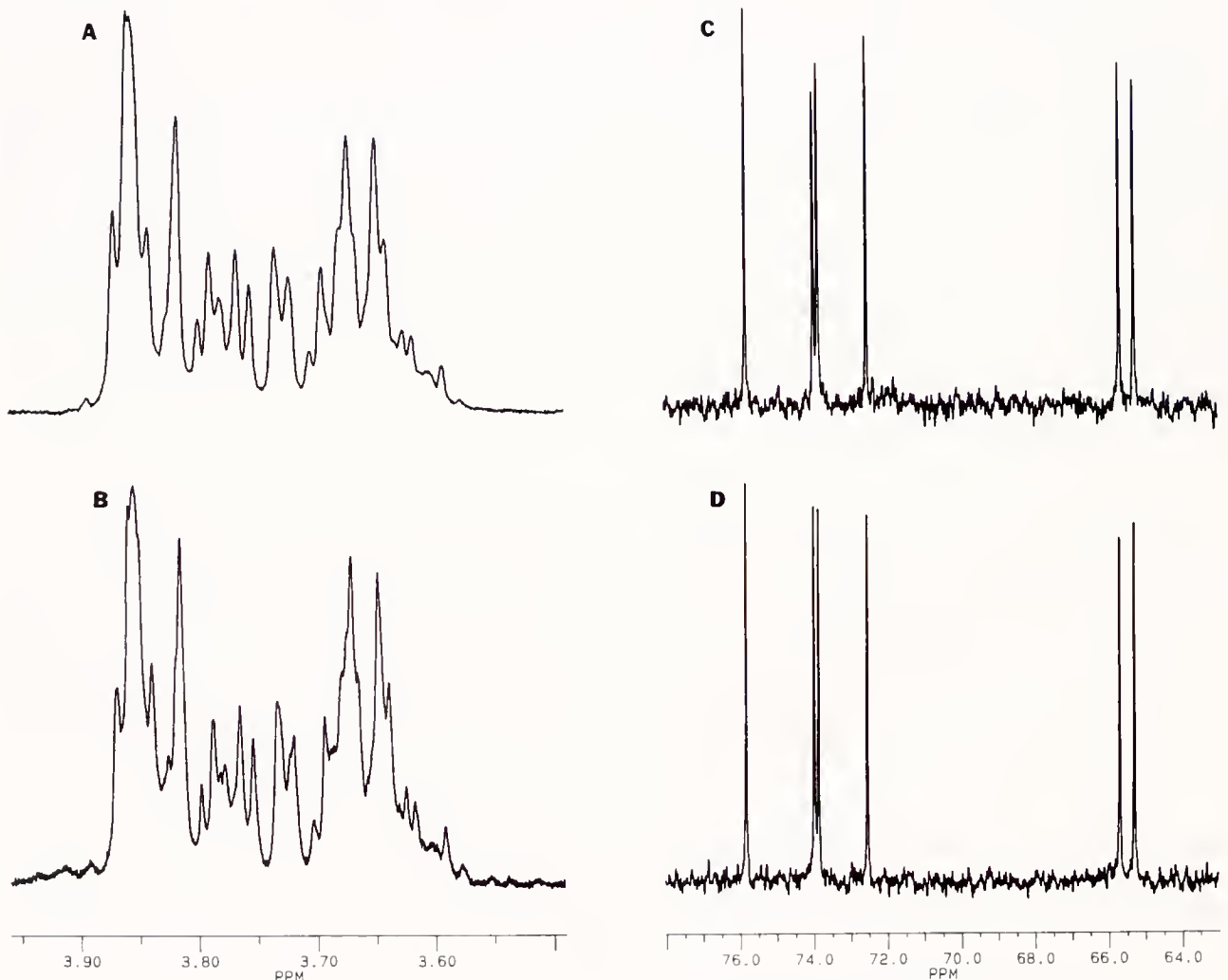


Figure 1. ^1H -NMR spectra of authentic sorbitol (A) and the ethanol-soluble fraction of *Eunapius fragilis* gemmules (B) and ^{13}C -NMR spectra of authentic sorbitol (C) and the ethanol-soluble fraction of *Eunapius fragilis* gemmules (D).

Table 1

¹³C-NMR chemical shifts measured downfield from the internal standard DSS in D₂O of authentic sorbitol and the ethanol soluble fraction from gemmules of *Eunapius fragilis*

Sample	¹³ C chemical shifts					
<i>E. fragilis</i> extract	65.3	65.7	72.5	73.8	74.0	75.8
Sorbitol	65.2	65.7	72.5	73.8	73.9	75.8

mules—from a maximum of about 36 $\mu\text{mol/gfw}$ to a steady-state level of about 4 $\mu\text{mol/gfw}$ by 36 h (Fig. 2). Sorbitol remained constant over the same time in gemmules maintained at 5°C. Concomitant with the decline in sorbitol, there was a significant increase in glycogen content measured by anthrone—from about 29 μmol hexose equivalents/gfw to about 62 μmol hexose equivalents/gfw (Fig. 3). As with the sorbitol, glycogen levels remained constant when gemmules were maintained at 5°C.

Enzyme activities

Sorbitol dehydrogenase activity was undetectable in the quiescent gemmules. During gemmule germination at 20°C, activity increased dramatically to a maximum of 0.2 $\mu\text{mol/min} \cdot \text{mg}$ protein at 36 h (Fig. 4). Between 36 and 48 h, the activity declined again, to about 0.05 $\mu\text{mol/min} \cdot \text{mg}$ protein. In contrast, the activity of aldose reductase remained at about 8 $\mu\text{mol/min} \cdot \text{mg}$ protein throughout germination (Fig. 4).

Total glycogen phosphorylase activity increased by a factor of 2.3 during germination—from a low of 1.6 to a high of 3.7 $\text{nmol/min} \cdot \text{mg}$ protein (Fig. 5). Maximal activity occurred by 42 h. The percentage of glycogen phosphorylase *a* declined from a maximum of 100% in quiescent gemmules to a minimum of 84% at 42 h. In contrast to changes in glycogen phosphorylase activity, there were no significant changes in the activity of either the glucose-6-phosphate-dependent or the glucose-6-phosphate-independent glycogen synthase during germination (Fig. 6). The activity of the glucose-6-phosphate-dependent glycogen synthase is 18 times greater than the activity of the glucose-6-phosphate-independent glycogen synthase. Note, however, that the total glycogen phosphorylase activity is three times higher than the glucose-6-phosphate-independent glycogen synthase activity and one-fourth of the activity of the glucose-6-phosphate-dependent glycogen synthase activity.

cAMP concentrations did not change significantly during the germination of gemmules of *Eunapius fragilis*, ranging from 3.6 ± 0.7 to 4.0 ± 0.8 pmol/mg protein.

Discussion

Germination of *Eunapius fragilis* gemmules under the conditions described here occurs during the first 42 to 50 h following exposure to 20–23°C. During that time, sorbitol levels decline dramatically and glycogen levels increase. It is possible that the sorbitol is being used to synthesize glycogen. Indeed, the changes in the two carbohydrates—measured as micromoles per gram of fresh

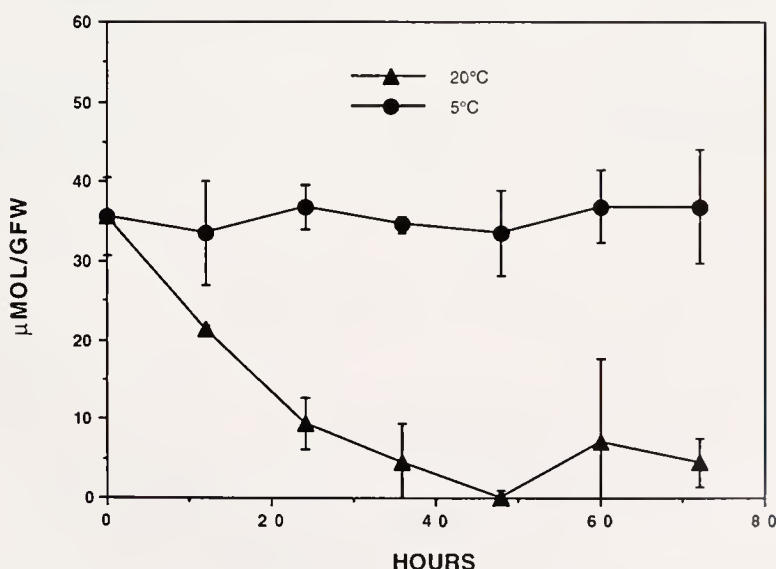


Figure 2. Changes in the levels of sorbitol in gemmules of *Eunapius fragilis* during germination. Values are expressed as means $\pm$ 1 SE ($n = 3$).

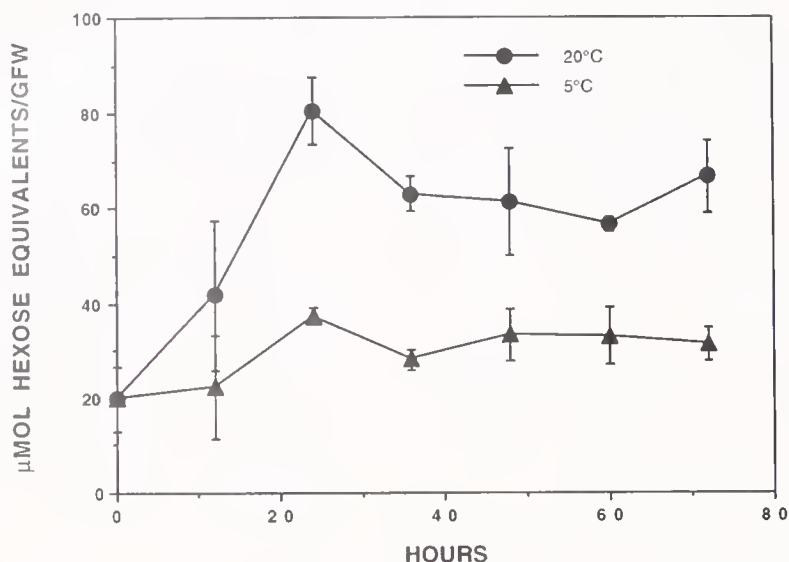


Figure 3. Changes in the levels of glycogen in gemmules of *Eunapius fragilis* during germination. Values are expressed as means $\pm$ 1 SE ($n = 3$).

weight of gemmule tissue ($\mu\text{mol/gfw}$)—balance one another very closely: sorbitol decreases by $32 \mu\text{mol/gfw}$ (from an average control value of $36 \mu\text{mol/gfw}$ to an average steady-state level of $4 \mu\text{mol/gfw}$), and glycogen increases by $33 \mu\text{mol/gfw}$ (from an average control value of $29 \mu\text{gfw}$ to an average steady-state value of $62 \mu\text{mol/gfw}$). The synthesis of glycogen from a carbohydrate precursor is consistent with observations for other develop-

ing invertebrates; for example, during the first few hours of germination, the osmotic pressure of gemmules of *Spongilla lacustris* drops precipitously (Rasmont, 1975). Rasmont (1975) attributed this reduction in osmotic pressure to the resynthesis of glycogen from an unidentified low molecular weight precursor. Also, in insect larvae, the synthesis of glycerol and sorbitol during exposure to cold occurs at the expense of glycogen, and

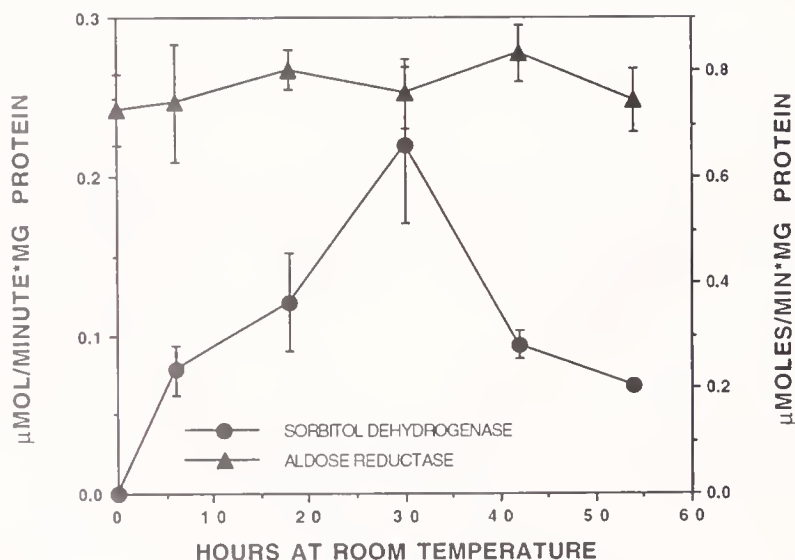


Figure 4. Changes in the activity of sorbitol dehydrogenase and aldose reductase in gemmules of *Eunapius fragilis* during germination. Assay conditions are as outlined in the Materials and Methods section. Values are expressed as means $\pm$ 1 SE ($n = 3$).

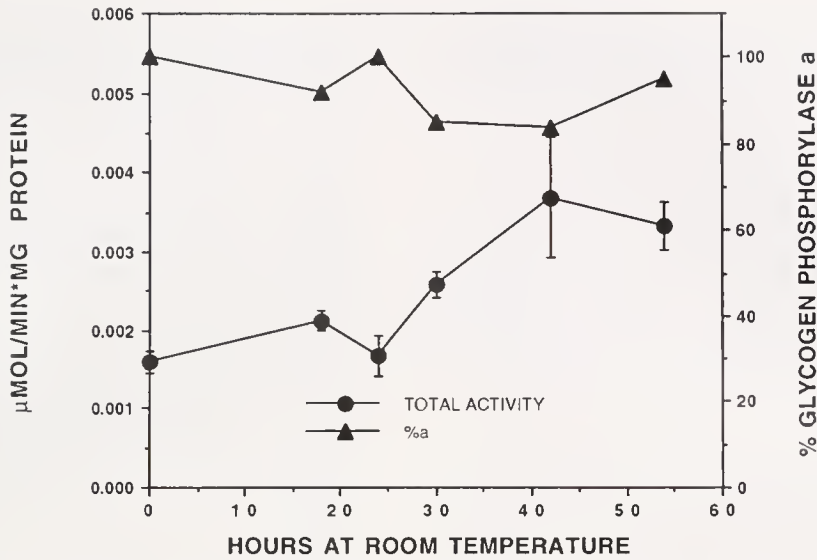


Figure 5. Changes in the activity of total glycogen phosphorylase and the percentage of glycogen phosphorylase *a* in gemmules of *Eunapius fragilis* during germination. Assay conditions are as outlined in the Materials and Methods section. Values are expressed as means $\pm$ 1 SE ($n = 3$).

during subsequent warming, glycogen levels are subsequently restored at the expense of sorbitol (Storey and Storey, 1983). Clegg (1964) reports that during the pre-emergence development of *Artemia salina* cysts, trehalose is utilized to synthesize glycogen and glycerol during development. In this case, the decrease in trehalose can account for all of the synthesis of glycogen and glycerol plus the oxygen consumption measured during pre-

emergence (Clegg, 1964). This is not the case for *Eunapius fragilis*, however. The amount of sorbitol breakdown can just account for the amount of glycogen synthesized. Calorimetric measurement of heat dissipation of the gemmules of *Eunapius fragilis* shows that the energy flow of gemmules increases dramatically during germination (Loomis *et al.*, 1996). Clearly, another source of energy must be available during germination. This

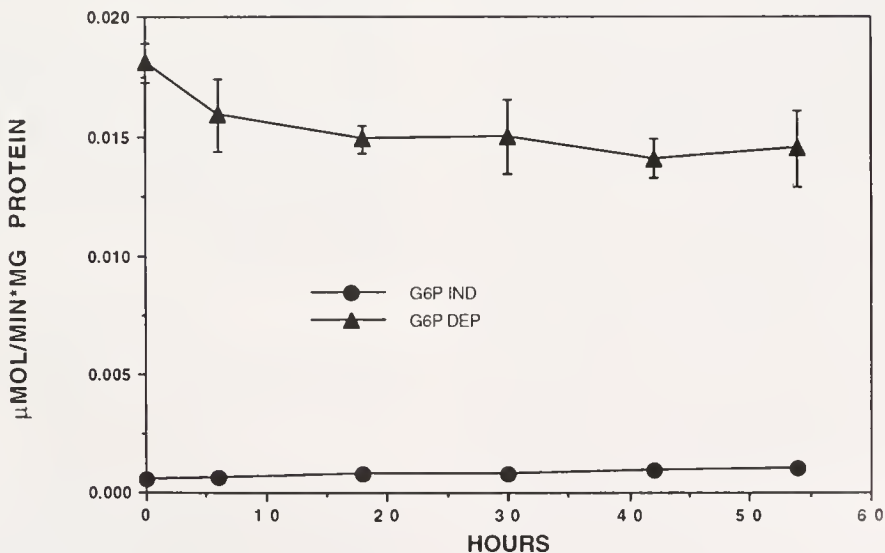


Figure 6. Changes in the activity of glucose-6-phosphate-dependent and glucose-6-phosphate-independent glycogen synthase in gemmules of *Eunapius fragilis* during germination. Assay conditions are as outlined in the Materials and Methods section. Values are expressed as means $\pm$ 1 SE ($n = 3$).

source of energy is most probably nutrients contained in yolk platelets. The thesocytes of gemmules are packed with yolk platelets, and these platelets begin to shrink about 24 h after the initiation of germination in *Spongilla lacustris* (Simpson, 1984).

Using histological techniques, De Vos (1977) has shown that glycogen is present in the early stages of gemmule formation and later disappears. This is consistent with the lower levels of glycogen measured in post-diapausing gemmules of *Eumapius fragilis*. It may be that the glycogen originally present is used to synthesize sorbitol during development of gemmules, although this has yet to be determined.

Sorbitol is a well-known cryoprotectant produced during the winter in insects (see Lee, 1989; Baust *et al.*, 1985, for reviews), and it probably imparts increased freezing and desiccation tolerance to gemmules of *Eumapius fragilis*. In fact, gemmules of *Eumapius fragilis* are remarkably tolerant to freezing and desiccation (Fell, 1990; Boutselis *et al.*, 1990). For example, post-diapausing, fully hydrated gemmules will germinate following a 30-day exposure to -72°C (Boutselis *et al.*, 1990), and they survive air-drying for several weeks (Fell, 1990). Increased tolerance to cold and desiccation is no doubt important for the survival of *Eumapius fragilis* since this species is found in environments that can dry in the summer (such as swamps in the southeastern United States) and freeze in the winter (such as ponds and streams in the northeastern United States).

To begin to understand the biochemical mechanisms controlling the breakdown of sorbitol and the synthesis of glycogen during germination in *Eumapius fragilis* gemmules, we measured the activities of the proximate enzymes responsible for catabolism and anabolism of each of these compounds. Of course such analyses do not rule out modulations in other auxiliary enzymes that may also contribute to the altered mobilization of carbohydrate stores.

Sorbitol breakdown may result from an increase in the activity of sorbitol dehydrogenase (the catabolic enzyme), a decrease in the activity of aldose reductase (the anabolic enzyme), or both. In *Eumapius*, sorbitol breakdown during the germination of gemmules appears to be the result of an increase in the activity of sorbitol dehydrogenase. The activity of this enzyme increases from undetectable levels in quiescent gemmules to more than $0.2\ \mu\text{mol}/\text{min} \cdot \text{mg}$ protein during the first 30 h of germination; during the same period, more than 95% of the sorbitol breakdown occurs. Aldose reductase activity remains unchanged throughout germination. Therefore, increased catabolic capacity could account for the observed changes in sorbitol levels during germination. The increase in maximal activity of the catabolic enzyme,

sorbitol dehydrogenase, could be the result of either *de novo* synthesis of the enzyme or catalytic activation *via* covalent modification during the early stages of germination; it is still unclear whether one or both of these processes are responsible.

Similarly, glycogen synthesis could result from an increase in the activity of glycogen synthase (the anabolic enzyme), a decrease in the activity of glycogen phosphorylase (the catabolic enzyme), or both. In *Eumapius*, there is no clear indication whether any of these possibilities occur. Glycogen synthase activity, whether dependent on or independent of glucose-6-phosphate, remains unchanged during germination. Total glycogen phosphorylase activity, on the other hand, increases during germination, but this increase is at least partly nullified by the conversion of some of the active glycogen phosphorylase *a* form to its inactive *b* form. Similarly, there are no changes in the concentration of cAMP (this paper), ATP or AMP (Loomis *et al.*, 1996) that could promote allosteric or covalent changes *in vivo* in glycogen phosphorylase and glycogen synthase. Indeed, the relatively high concentrations of cAMP may keep most of the glycogen synthase in the glucose-6-phosphate-dependent form. These data are in contrast with results from *Spongilla lacustris* gemmules in which cAMP levels are dramatically reduced during the first 2 h of exposure to 20°C (Simpson and Rodan, 1976). Simpson (1984) has suggested that the reduction is due to the combination of a decrease in adenylate cyclase activity and an increase in cAMP phosphodiesterase activity. In *Spongilla*, the low levels of cAMP could result in a decrease in the breakdown of glycogen through the inhibition of glycogen phosphorylase.

Clearly, other control mechanisms that do not involve changes in cAMP must be responsible for the synthesis of glycogen in gemmules of *Eumapius fragilis*. It is possible that changes in the concentration of glucose-6-phosphate during germination could increase the rate of glycogen synthesis by allosterically activating the glucose-6-phosphate-dependent glycogen synthase. We are examining this possibility.

Acknowledgments

This work was supported by NSF grants DCB-9018579 and IBN 9306652 to SCH.

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Sources of Energy for Increased Metabolic Demand During Metamorphosis of the Abalone *Haliotis rufescens* (Mollusca)

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Abstract. Pelagic, lecithotrophic (nonfeeding) larvae of the red abalone (*Haliotis rufescens*) settle and subsequently metamorphose into benthic juveniles capable of feeding on particulate food. Thus, metamorphosis must be fueled by either endogenous reserves or a nonparticulate food source such as dissolved organic material (DOM) in seawater. The metabolic rates (measured as oxygen consumption) of abalone larvae were found to increase by an average of 3- to 5-fold from the larva to early juvenile stage. The total cost of development from embryo to juvenile measured for three cultures ranged from 41.6 mJ to 55.0 mJ. Meeting this cost would require 1.3 to 1.7 μg of biomass (ash-free dry mass), which is similar to the initial biomass of the spawned oocyte at $1.36 \pm 0.04 \mu\text{g}$ (mean of four cultures). However, there was no net loss of biomass during development from the oocyte to the juvenile. The uptake of alanine and glucose from seawater by larvae and juveniles could provide one-third of the organic material required to supply metabolism, even if the transporters were only operating at 20% of their maximum capacity throughout development. For larvae undergoing metamorphosis (between 6- and 9-days-old) the proportion of total metabolic demand supplied using aerobically catabolized biomass was only 39%. The higher metabolic rates of metamorphosis are met only in part by consuming stored endogenous reserves. Concomitant with an increase in mass-specific

metabolic rate during metamorphosis, the maximal capacity (J_{max}) for the transport of dissolved alanine from seawater increased 3-fold, from 61.2 ± 1.9 (SE) to 182.0 ± 49 pmol alanine individual⁻¹ h⁻¹. The majority (range: 61% to 100%) of the energy requirements of larval and early juvenile development of *H. rufescens* could be supplied by input of DOM from the environment. Measurements of transport rates of amino acids and sugars by these animals, and calculations of the energy input from these substrates, indicate that the cumulative transport of DOM from seawater during development to the early juvenile stage could supply an amount of energy equivalent to the initial maternal endowment of energy reserves to the oocyte of this lecithotrophic species.

Introduction

Metamorphosis of marine invertebrates from a pelagic larva to a juvenile can involve reorganization of existing tissue and construction of new tissue (*e.g.*, ascidians: Cloney, 1961; echinoderms: Hinegardner, 1969). For many species, the processes involved in metamorphosis take place after the larva has settled to the benthos in response to environmental cues (Hadfield, 1986). For example, haliotid larvae settle on encrusting red algae (Morse and Morse, 1984) and subsequently metamorphose over the next 2 to 7 days into juveniles (Crofts, 1937). During this time, they lose their velum, develop enlarged gills and foot, and begin deposition of the adult shell. The rearrangement of tissues at metamorphosis is potentially energetically costly and may result in depletion of endogenous reserves. Lucas *et al.* (1979) reported that metamorphosing barnacle cyprids (*Balanus bala-*

Received 24 August 1995; accepted 10 September 1996.

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noides) showed increases in metabolic rates while energy reserves (protein and lipid) decreased. Similarly, newly settled oyster larvae (*Ostrea edulis*) used endogenous lipid reserves (Holland and Spencer, 1973), or lipid and protein (Rodriguez *et al.*, 1990), in the transition from larva to settled spat. It is still unclear, however, to what extent larval energy reserves affect post-metamorphic success (Highsmith and Emler, 1986; Pechenik and Eyster, 1989).

The rate of depletion of energy reserves for embryos and larvae of several species of marine invertebrates is insufficient to meet total metabolic demands during development; although sufficient energy is available in the egg, these reserves are not used (Jaekle and Manahan, 1989a; Shilling and Manahan, 1990). The conclusion from these studies was that uptake of DOM from seawater was providing the missing energy. The reports to date of the physiology of metamorphosis of marine invertebrates have focused mainly on the role of endogenous reserves (*e.g.*, Holland and Spencer, 1973; Rodriguez *et al.*, 1990). However, bivalves undergoing metamorphosis can absorb dissolved amino acids from seawater during metamorphosis (Manahan and Crisp, 1983), although the quantitative importance of this process to metabolism is unknown. Haliotid larvae cannot feed on particles (Crofts, 1937) but do have the capacity to transport dissolved organic material (DOM, *e.g.*, amino acids) from seawater (Jaekle and Manahan, 1989b) and do not use endogenous reserves to meet the total requirements of metabolism (Jaekle and Manahan, 1989a). These observations lead to the suggestion that DOM could be important to larval energetics. In the present study, we address three questions: (1) What are the metabolic costs during complete metamorphosis of abalone larvae (*H. rufescens*)? (2) What proportion of these costs can be met through utilization of endogenous reserves? (3) How much energy does exogenous DOM contribute to the energetics of metamorphosis?

Materials and Methods

Culturing of larvae and plantigrades

Batches of fertilized abalone oocytes, from seven separate spawnings, were cultured from the zygote to up to 6 days after settlement (juvenile) in flowing 5- μ m-filtered, UV-irradiated seawater at a commercial abalone hatchery (the AbLab, Port Hueneme, CA). The temperatures at which the larvae were reared, and the corresponding temperatures used for physiological measurements, varied with time of year. The following is a list of the cultures by letter designation, with month and temperature ($\pm 1^\circ\text{C}$): Culture A—March, 12°C ; B—May, 13°C ; C—June, 15°C ; D—July, 20°C ; E—February, 13°C ; F—March, 12°C ; G—May, 15°C . Measurements

of ash-free dry weight (biomass) and all physiological measurements were not done on all stages or for all seven cultures (specifics given in Results). All stages were maintained on 80- μ m-mesh screens that were immersed in the running seawater. Veliger-stage larvae were induced to settle with γ -amino butyric acid (GABA, Morse *et al.*, 1979) once they had attained metamorphic competency (5 to 9 days post-fertilization). Under the conditions in use in the commercial abalone hatchery at the time of our experiments, a concentration of 100 μM GABA with a 30-min exposure was used to induce metamorphosis.

The stages of larvae and plantigrades (early post-settlement) used were characterized according to various morphological attributes (*e.g.*, presence of velum). Live animals were observed under dissecting and compound microscopes. The stages were defined as follows: 0—unfertilized oocyte; i—veliger not competent to settle; ii—swimming veliger competent to settle, has velum, branched cephalic tentacles, eyes, and the capacity to crawl briefly on its foot; iii—newly settled larva no longer swimming (referred to as a plantigrade), but with similar morphology to stage ii; iv—metamorphosing plantigrade, which has lost velum, adult shell is growing from edge of larval shell, gill buds are apparent, mouth parts are developing, and foot is larger. Stage ii is equivalent to stage 21 as defined by Hahn (1989) for *H. rufescens*, and stage iv is equivalent to stage 4 (for plantigrades) for *H. discus hannai* (Hahn, 1989).

The possibility that plantigrades (stage iii) and juveniles (stage iv) were feeding on particulate material was assayed by placing the animals on a glass slide coated with diatoms and bacteria and observing the animals with a compound microscope. When present, the mouth and radula were readily visible, as were the feeding tracks left by the feeding juvenile. All settled stages were tested in this way. The slides were coated by placing them either in ambient, continuously running, unfiltered seawater for several days or in the presence of cultured unialgae (*Thalassiosira pseudonana*, *Dunaliella tertiolecta*, and *Rhodomonas* sp.).

Measurement of metabolic rates

Oxygen consumption was measured for swimming larvae and settled plantigrades using coulometric respirometry. This technique allows long-term, continuous measurement of oxygen consumption rates by replacing the oxygen as it is depleted from the respiration chamber. The apparatus and methods used were those of Heusner *et al.* (1982) that have been adapted for use with marine invertebrate larvae (Hoegh-Guldberg and Manahan, 1995). Glass respirometry chambers were sterilized with 70% ethanol and rinsed thoroughly with sterile-autoclaved seawater. Swimming larvae or crawling planti-

grades were rinsed with autoclaved seawater and placed into the respirometry chambers in 1 to 2 ml of filtered ($0.2\text{-}\mu\text{m}$ pore-size), autoclaved seawater (the volume of seawater does not affect the measurement). The number of individuals per chamber ranged from 22 to 911 (larvae) and 44 to 243 (plantigrades). The measurement of absolute rates of oxygen consumption by larvae of marine invertebrates is not affected by larval densities in the ranges used in the present study (Hoegh-Guldberg and Manahan, 1995). After equilibration to temperature and pressure (1 to 4 h), recording commenced and was continued for 6 to 30 h. The seawater within the chambers was not stirred. The basis of the coulometric respirometry technique does not require stirring for accurate measurement of oxygen consumption (Heusner *et al.*, 1982). Each set of measurements was conducted at the temperatures at which the larvae and plantigrades were cultured. One respiration chamber containing seawater, but without animals, was run in parallel as a control for oxygen depletion that was not due to the presence of the animals. Jaeckle and Manahan (1989b) found very few bacteria attached to abalone larvae (<6 per larva) using epifluorescence and scanning electron microscopy, thus the contribution of bacterial metabolism to measured respiration rates is probably below the limit of detection. About every 24 h the animals in the chambers were replaced with new animals from the same culture, at which time the chambers were cleaned again. New animals were used to reduce differences in metabolism that might exist between the cultured animals (used for biomass and nutrient transport determinations) and those maintained in the respirometer. At the end of each time period the animals in each chamber were removed and counted to allow for the calculation of metabolic rates on a per-animal basis.

A second technique for measuring rates of oxygen consumption was also used, the Winkler titration method (Parsons *et al.*, 1984). This involved measuring the depletion of oxygen in a sealed BOD (biological oxygen demand) bottle (temperature = 15°C) that contained swimming veligers (stage ii, Culture G, 7-day-old). Six BOD bottles (volume = 300 ml each) were filled with larvae suspended in filtered seawater ($0.2\text{-}\mu\text{m}$ pore-size) with densities ranging from 651 to 1987 larvae per bottle. One bottle containing only filtered seawater (control) was incubated for 6 h, as were the bottles containing larvae. The seawater within the bottles was not stirred during incubation. All of the oxygen in the bottles was then chemically fixed and the Winkler titration conducted. The larvae in each bottle were removed and counted immediately after the chemical fixing and measurement of oxygen was complete. The larval shells and attached tissue were easily visible after the chemical fixation for Winkler titration.

Biomass determination (ash-free dry weight)

Net changes in total biomass per individual were measured to determine how much of the metabolic cost was met by depletion of endogenous reserves. Six replicate samples per day were taken for each culture for biomass determination. Biomass of the various developmental stages was determined as described by Jaeckle and Manahan (1989a). Briefly, the organisms were washed with filtered ($0.2\text{-}\mu\text{m}$ pore-size) 3.4% ammonium formate (w/v), to replace sea-salts, placed in an aluminum dish, and dried at 80°C . The dried tissue was weighed, ashed at 450°C for 5 to 6 h, and weighed again. For a given culture, all sampled stages of development were stored frozen (-20°C) until they were dried, ashed, and weighed together. This was done to reduce any effects from the drying and ashing treatments on comparisons among stages for a given culture. The difference between the ash weight and dry weight is the ash-free dry organic weight, here defined as biomass. The changes in biomass were converted into energy by using the enthalpic equivalents given by Gnaiger (1983). Comparisons between mean biomasses were conducted using analysis of variance (ANOVA; Zar, 1984). When a difference was observed using ANOVA within a culture, a multiple comparison test (Newman-Keuls) was conducted to permit comparisons among biomasses for different days. Errors are presented either as standard error of the mean (SEM) or 95% confidence intervals (95% CI).

Measurement of amino acid and sugar transport rates

The kinetic constants for glucose and alanine transport were measured for swimming larvae and crawling plantigrades to determine what proportion of the metabolic costs during metamorphosis could potentially be supplied by transport of exogenous dissolved amino acids and sugars. The substrate concentrations used for these kinetic experiments ranged from 0.1 to $100\text{-}\mu\text{M}$, with 50 to 300 animals being exposed to radioactively labeled substrates in 2 ml of sterile-filtered seawater. Measurements were conducted at the temperatures at which the organisms were cultured. A technique for measuring rates of transport was employed which was very similar to that used by Manahan (1983) for other molluscan larvae, where animals were exposed undisturbed for a short time to a known concentration of substrate added to seawater. This technique was used in the present study because the periodic disturbances of late-stage abalone larvae associated with other transport assays used for larvae (*e.g.*, Jaeckle and Manahan, 1989b; Shilling and Manahan, 1994) caused them to cease swimming for as much as 30 s. This might have resulted in underestimates of transport rates. A potential problem with single, end-point rate determinations is that the zero

time point may have a positive y-intercept due to radioactivity retention not associated with transport. In the transport measurements described below, this possible artifact was accounted for by subtracting the radioactivity retained due to nonspecific binding to the animals and filters.

Transport of ^3H -labeled alanine and ^{14}C -labeled glucose (2 or 5 μCi per 10 ml; glucose: 353 mCi mmol $^{-1}$, alanine: 84 Ci mmol $^{-1}$, New England Nuclear) from seawater was measured by exposing the animals to radiolabeled substrate for a known time (10 to 15 min). The animals were then separated from the radioactive medium using vacuum filtration onto a Nuclepore membrane filter (5 μm pore-size). The animals were washed three times with 5 to 10 ml (each wash) of seawater and were vacuumed to dryness on the filter between each wash. As controls, filters without animals and filters with heat-killed animals (65°C for 10 min) were exposed to radiolabel and washed in an identical fashion. The amount of radiolabel retained by the filter alone, or the filter and dead animals, was always less than 10% of that retained by the filter and live animals, and was subtracted from the total amount of radioactivity on the filter with live animals. The animals on the filter were counted using a dissecting microscope and placed in tissue-solubilizer (Scintigest, Fisher Scientific) for 24 h, after which scintillation cocktail (Bio-HP, Fisher Scientific) was added for liquid scintillation counting. After appropriate quench correction and calculations to determine the moles of substrate transported per individual, affinity (K_s) and maximal transport capacity (J_{max}) were calculated for alanine and glucose using Eadie-Hofstee transformations of transport rate.

Results

Rates of oxygen consumption before and during metamorphosis

Metabolic rates (as oxygen consumption) were determined continuously using coulometric respirometry over periods of days for veligers and plantigrades from three cultures (Fig. 1 and Table I). Swimming veligers (stage ii) had mean metabolic rates ($\pm 95\%$ CI) ranging from 197 (± 34) to 347 (± 40) pmol O $_2$ individual $^{-1}$ h $^{-1}$ for 1 or 2 days prior to settlement (Cultures E and G, respectively; Table I). The mean metabolic rate for stage ii larvae was 292 (± 9) pmol O $_2$ individual $^{-1}$ h $^{-1}$ for Cultures C, E, and G. Variation among metabolic rates for animals from different cultures may in part be due to the difference in culturing temperature (see Methods) and also batch-to-batch variation (Fig. 3, Jaecle and Manahan, 1989a). For all three cultures there was a significant increase in metabolic rate coincident with the time when metamorphosis into the juvenile was occurring (e.g.,

Culture C: stage iii, 222 ± 10 pmol O $_2$ individual $^{-1}$ h $^{-1}$ to stage iv, 1659 ± 59 pmol O $_2$ individual $^{-1}$ h $^{-1}$, Table I; Fig. 1). During this period there was no observable increase in crawling activity, as seen under dissecting and compound microscopes. Plantigrades at stage iv (3 to 4 days post-settlement) were undergoing morphological changes associated with metamorphosis (see Methods section for description of stages).

Metabolic rates of veliger larvae (stage ii, 7-day-old) from Culture G were also determined (in addition to coulometric measurements) by measuring depletion of oxygen from seawater in six sealed 300-ml BOD bottles. The rates measured ranged from 173 pmol oxygen larva $^{-1}$ h $^{-1}$ to 261 pmol oxygen larva $^{-1}$ h $^{-1}$, with a mean rate of $213 (\pm 35, 95\% \text{ CI})$ pmol larva $^{-1}$ h $^{-1}$ for the six independent measurements. This rate measured at 15°C is less than that for sibling stage ii larvae measured using the coulometric respirometry method (Table I, Culture G, 288 ± 34 pmol oxygen larva $^{-1}$ h $^{-1}$). Both methods gave significantly higher respiration rates than previously reported values obtained using polarographic oxygen sensors (see Discussion).

Changes in biomass before and during metamorphosis

Changes in biomass for five cultures of abalone are shown in Figure 2; an asterisk above a bar represents a significant ($P < 0.05$) difference between the biomass on the stage so marked and that of the day or stage before. In two of the four cultures for which there was an oocyte measurement (Cultures A and D), there was no significant difference (ANOVA: $P > 0.10$) between the biomass of competent veligers (stage ii) and that of the oocytes (stage 0). In both of the other two cultures (Cultures B and C) the veligers had a lesser biomass than the oocytes (Newman-Keuls: $P < 0.05$); for example, in Culture C the veligers (stage iib) lost 17% ($0.24 \mu\text{g}$) of their biomass relative to the oocytes. During early post-settlement (stage iii, first 2 days), newly settled plantigrades in four of five cultures showed an increase in biomass at some point before metamorphosis had commenced (Fig. 2, Newman-Keuls: $P < 0.05$). These changes occurred either between stages ii and iii or between 2 consecutive days at stage iii (labeled in Fig. 2 as iiia and iiib, respectively). During this time most of the animals still had their vela and were crawling, and by the second day post-settlement the adult shell was beginning to develop. No feeding activity was observed during this time. Of the four cultures (B, C, D, and G), in which the plantigrades (to stage iv) were cultured through metamorphosis, only one (culture B) showed a statistically significant (Newman-Keuls: $P < 0.05$) decrease in biomass from stage iii ($1.63 \pm 0.049 \mu\text{g}$) to stage iv ($1.37 \pm 0.038 \mu\text{g}$). In all three of the cultures (B, C, and D) for which data were

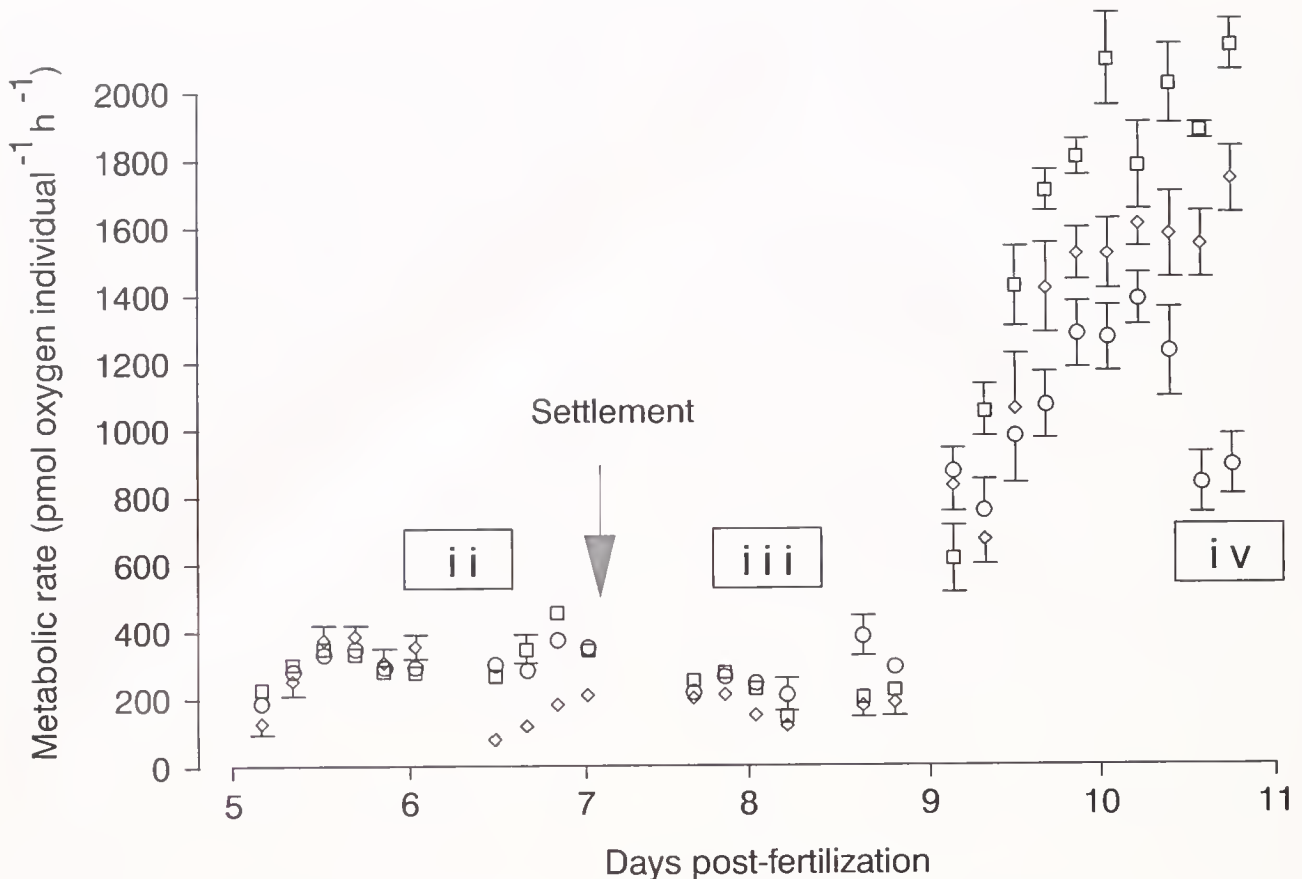


Figure 1. Rates of oxygen consumption of larvae and plantigrades from Culture C. Each symbol represents the mean of twelve 20-min rate measurements, for a total of 4 h. Error bars represent 1 standard error of the mean. Different symbol types represent separate respiration chambers. The gaps between runs represent times when the animals were being replaced with new animals and the chambers cleaned. "Settlement" indicates the time when larvae were treated with γ -amino butyric acid at the AbLab (see Methods). The boxed roman numerals indicate the stage of development (see Methods for definitions).

available to compare oocytes and plantigrades (stage 0 to iv, respectively), there were no significant differences between the oocytes' biomasses and those of the plantigrades (ANOVA: $P > 0.10$).

Meeting metabolic costs with endogenous reserves

Can newly settled plantigrades meet their metabolic needs through catabolism of endogenous tissue reserves? In Table II the energy required to meet metabolic needs was compared to the energy available from changes in aerobically catabolized biomass. The two most important energy reserves for abalone larvae are protein and lipid, total carbohydrate content being below the level of detection (Jaekle and Manahan, 1989a). Because we do not know how protein and lipid fluctuate during metamorphosis, the value (31.5 kJ g^{-1}) used in Table II to convert biomass to energy was the mean of the values for protein (24.0 kJ g^{-1}) and lipid (39.5 kJ g^{-1} ; Gnaiger,

1983). Similarly, the enthalpy of combustion value used for oxygen ($484 \text{ kJ (mol oxygen)}^{-1}$) was the mean of the values for lipid combustion ($441 \text{ kJ (mol oxygen)}^{-1}$) and protein combustion ($527 \text{ kJ (mol oxygen)}^{-1}$; Gnaiger, 1983). Culture C was chosen for these calculations because it had the most complete data set for each developmental stage's metabolic rate and biomass. Developing stages from Culture C (Table II) had 6.35 mJ of available energy for the transition from stage ii (day 6) to stage iv (day 9). The required energy for the same period, calculated from metabolic rates and gains in biomass, was 16.41 mJ. Thus the settled plantigrades could account for only 38.7% of their energy requirements using endogenous reserves.

The biomasses of the oocytes (from Cultures A, B, C, and D) ranged from 1.24 to $1.44 \mu\text{g}$ with a mean of $1.36 (\pm 0.038) \mu\text{g}$ (Fig. 2). The hatching of the bars for Culture C (Fig. 2) represents the expected loss of energy as biomass that would be needed to meet the measured metabolic

Table I

Rates of oxygen consumption of larvae and plantigrades before and during metamorphosis

	Days post-fertilization							
	6	7	8	9	10	11	12	13
Culture C (15°C): [stage]	[ii]	[ii]	[iii]	[iv]	[iv]			
Metabolic rate								
(pmol oxygen individual ⁻¹ h ⁻¹)	307 (12)	302 (18)	222 (10)	705 (69)	1659 (59)			
Total oxygen (oocyte to juvenile)	= 113.52 nmol							
Equivalent energy	= 54.95 mJ							
Equivalent biomass	= 1.73 µg							
Culture E (13°C): [stage]				[ii]	[iii]	[iii]	[iv]	[iv]
Metabolic rate								
(pmol oxygen individual ⁻¹ h ⁻¹)				197 (34)	139 (38)	259 (101)	642 (97)	773 (93)
Total oxygen (oocyte to juvenile)	= 86.07 nmol							
Equivalent energy	= 41.66 mJ							
Equivalent biomass	= 1.31 µg							
Culture G (15°C): [stage]	[ii]	[ii]	[iii]		[iv]	[iv]		
Metabolic rate								
(pmol oxygen individual ⁻¹ h ⁻¹)	347 (40)	288 (34)	251 (24)		425 (73)	911 (184)		
Total oxygen (oocyte to juvenile)	= 103.07 nmol							
Equivalent energy	= 49.89 mJ							
Equivalent biomass	= 1.57 µg							

Each value is the mean hourly rate for a given day, with at least 80 measurements (at 20-min intervals, 2 or 3 chambers) used to determine a given mean. The values in parentheses are the 95% confidence intervals of each mean. The "total oxygen" values were calculated by multiplying each hourly rate by 24 h, then adding the daily rates for all of the days from oocyte [stage 0] to early juvenile [stage iv]. The rates for stages prior to the competent veligers (stage ii) were assumed to be the same as the stage ii veliger (see Jaeckle and Manahan, 1989a) and were included in the total oxygen calculation by multiplying by the age (in days). The total oxygen was multiplied by the mean oxyenthalpic equivalent of lipid and protein (484 kJ (mol oxygen)⁻¹) to obtain the "equivalent energy." This number was in turn divided by the enthalpic equivalent for aerobically catabolized biomass [(average values for protein and lipid (31.75 kJ g⁻¹)] to obtain the "equivalent biomass" needed to meet the metabolic cost. All enthalpic equivalents are from Gnaiger (1983).

needs (from Table I). This decrease in biomass was calculated by converting the measured rate of oxygen consumption for a given day (Table I) into a biomass-equivalent (calculated using values from Gnaiger, 1983). This biomass value was in turn subtracted from the calculated biomass of that day to give the expected biomass for the subsequent day. The equivalent biomass that would be needed to meet the calculated metabolic costs for the entire development, from oocyte to juvenile, ranged from 1.31 to 1.73 µg (Cultures E and C, respectively, Table I). These results are striking as they mean that *all* of the initial energy reserves (biomass) in the oocyte are needed for development, but none are used, as is evident from a comparison of oocyte and juvenile biomasses.

Rates of alanine and glucose transport during metamorphosis

Plantigrades (at 20°C, Culture D) 3 days after settlement (stage iv) had a 3-fold higher maximal transport capacity for alanine ($J_{\max} = 182.0 \pm 49.2$ [SE] pmol individual⁻¹ h⁻¹, $K_t = 96$ µM) than that of the stage ii veligers ($J_{\max} = 61.2 \pm 1.9$ pmol individual⁻¹ h⁻¹, K_t

$= 29$ µM) (Fig. 3A, B). In contrast to amino acid transport, the capacity for glucose transport decreased from stage ii (28.7 ± 5.1 pmol individual⁻¹ h⁻¹, $K_t = 19$ µM) to stage iv (14.5 ± 3.0 pmol individual⁻¹ h⁻¹, $K_t = 27$ µM) (Fig. 3C, D). A similar full kinetic analysis showed an increase for plantigrades of Culture F (at 12°C) where the $J_{\max}$ for alanine increased 2-fold from 27.9 ± 16.7 pmol individual⁻¹ h⁻¹ for stage iii to 51.5 ± 6.1 pmol individual⁻¹ h⁻¹ for stage iv (data not shown as a figure). These increases were concomitant with both the morphological changes and increase in metabolic rates associated with metamorphosis.

Discussion

Maternally endowed reserves in the egg, or reserves accumulated during the development of subsequent feeding larval forms, have been considered to be the sole source of energy for marine invertebrates during nonfeeding stages of development (e.g., metamorphosis of the barnacle: Lucas *et al.*, 1979; the bivalve: Holland and Spencer, 1973; Rodriguez *et al.*, 1990). However, studies with the bivalve *Crassostrea virginica* showed that stages undergoing

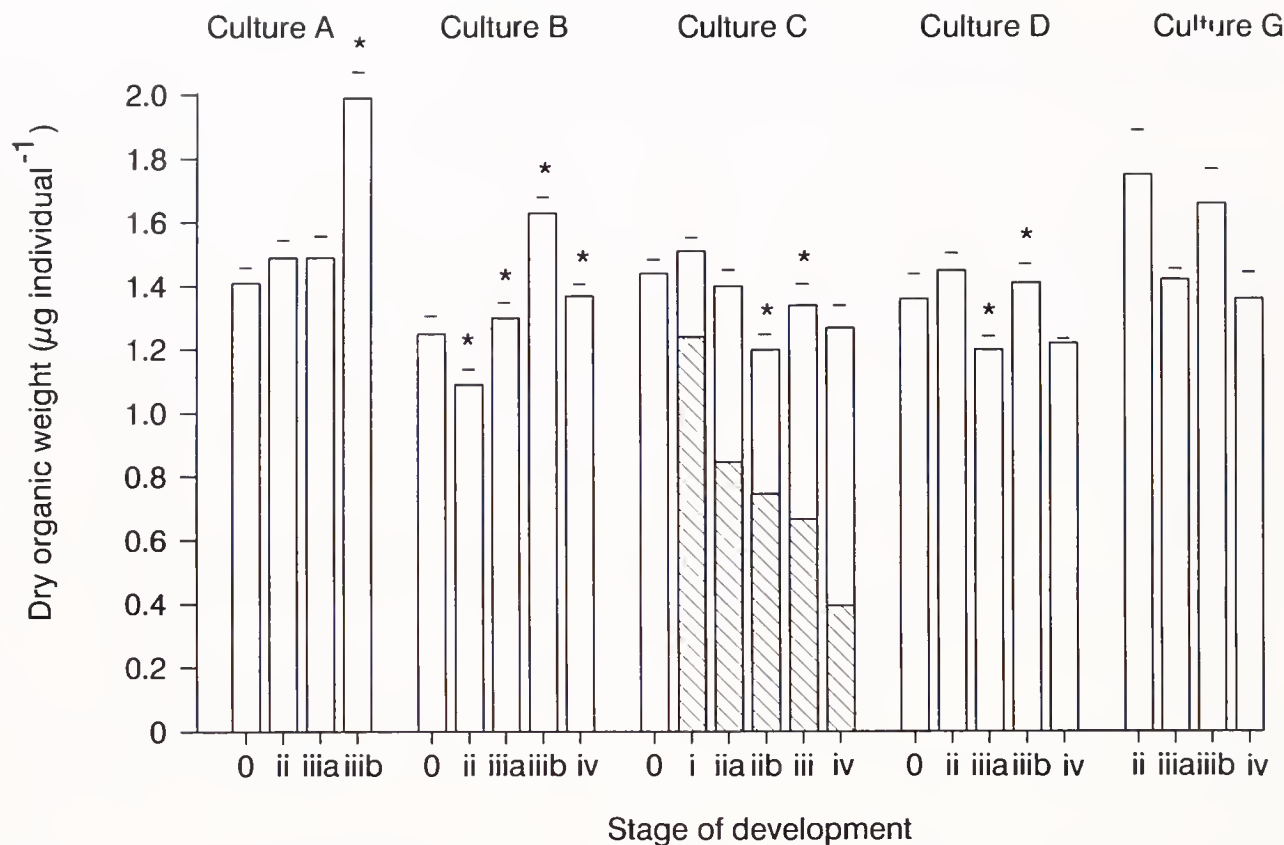


Figure 2. Biomass changes for cultures A, B, C, D, and G, from oocyte or veliger to plantigrade. Stages of development (0 to iv) are indicated on the x-axis and are described in the Methods section. Use of 'a' and 'b' after a stage represents a second sample (on another day) taken of the same stage. The line above each bar represents 1 standard error of the mean. The values for bars indicated by the asterisks (*) are significantly different ($P < 0.05$) from the values for bars of the stage immediately prior to them. For example, for Culture B stage ii is significantly lower than stage iii. The hatching of the bars for Culture C indicates the expected decrease in biomass due to aerobic catabolism of endogenous organic reserves (rates of oxygen consumption values from Table 1, conversion factors from Gnaiger, 1983).

metamorphosis can feed on phytoplankton (Baker and Mann, 1994). "Feeding" during bivalve metamorphosis can also occur *via* uptake of dissolved organic material (DOM) (Manahan and Crisp, 1983). Previous studies (Jaeckle and Manahan, 1989a) with the lecithotrophic larvae of *Haliotis rufescens* showed that depletion of endogenous reserves did not account for the energy requirements during larval development. In the present study, we confirm that finding and extend the conclusion of energy imbalance to stages undergoing metamorphosis from larva to juvenile. Only a small fraction of the total cost of larval development and metamorphosis could be accounted for by depletion of endogenous reserves (as measured by changes in dry organic mass, Fig. 2). There was no statistically significant loss of biomass from the oocyte to the juvenile stage for the cultures studied, even though at least 75% of the oocytes' biomass should have been used to account for metabolic cost of development to the juvenile stage (Fig. 2, Culture C shaded areas).

Metabolic costs of metamorphosis

There was an increased metabolic cost associated with metamorphosis of *H. rufescens* (Table 1). At about 2 days after induction of settlement, the plantigrade stages had an increased metabolic rate (day 9) compared to earlier stages of swimming veligers and newly settled plantigrades. This increase in metabolism continued until the measurements were discontinued at day 11 (Fig. 1, Culture C). There was no corresponding increase in biomass for stages of Culture C during this period of development (Fig. 2). One possible explanation for the increase in metabolic requirements is that it is energetically expensive to rearrange existing tissue and construct new ones, and that the observed increase in metabolic rate may reflect the energy cost of metamorphosis to the juvenile stage. Regardless of mechanism, increases in metabolic rates have been measured in other species undergoing tissue rearrangement (sea urchin embryos, Immers and Runn-

Table II

The contribution of endogenous reserves (biomass) to metabolic demands during metamorphosis for Culture C

	Days post-fertilization		
	6–7	7–8	8–9
Changes in biomass:			
biomass (μg)	–0.20	+0.41	0
equivalent energy (mJ) (31.75 kJ g^{-1})	–6.35	+4.45	0
Sum of available energy (mJ) (from decrease in biomass)	= –6.35		
Metabolic demand:			
metabolic rate (pmol d^{-1})	7308	6288	11,124
equivalent energy for days 7 to 9 (72-h period) (mJ) (484 kJ mol^{-1})	+3.54	+3.04	+5.38
Sum of required energy (mJ)	= +16.41		
gain in biomass = +4.45			
plus metabolic rate = +11.96			
Energy balance: Ratio of available to required energy	38.7		

Biomass values are from Figure 2; metabolic rates are calculated from those in Table I. The rates given per day are the average hourly means for the days encompassed by the period (e.g., “6–7” refers to days 6 and 7), multiplied by 24 h; for example, the metabolic rate in the period “6–7” is the average of the rates on days 6 and 7. Enthalpies of combustion used to convert changes in biomass to “equivalent energy” and the oxyenthalpic equivalent are from Gnaiger (1983, as used in Table I). “+” refers to gains in biomass or required energy for metabolism; “–” refers to losses in biomass. Stages of development corresponding to age in days are given in Table I and Figure 1.

strom, 1960; metamorphosing barnacles, Lucas *et al.*, 1979).

The values for rates of oxygen consumption presented here for larvae of *H. rufescens* are higher than previously reported (Jaeckle and Manahan, 1989a). In that study the rate of oxygen consumption measured with a polarographic oxygen sensor was $84 \text{ pmol oxygen larva}^{-1} \text{ h}^{-1}$, compared to the present values of 197 to $347 \text{ pmol oxygen larva}^{-1} \text{ h}^{-1}$, measured with coulometric respirometry, and $213 \text{ pmol oxygen larva}^{-1} \text{ h}^{-1}$, measured with Winkler titration. Concerns about the accuracy of polarographic oxygen sensors for measurements of larval metabolic rates are discussed in detail elsewhere (Hoegh-Guldberg and Manahan, 1995). Note, however, that the higher metabolic rates now measured for larvae of *H. rufescens* do not negate the earlier conclusion of Jaeckle and Manahan (1989a) that endogenous reserves do not fuel metabolic demands and that an exogenous energy source (*i.e.*, DOM in seawater) contributes to metabolic costs. Given higher values for metabolic rates, the calculated contribution from endogenous reserves would be even lower than estimated by Jaeckle and Manahan (1989a). Nor does the use of the lower value for metabolic rate ($84 \text{ pmol oxygen larva}^{-1} \text{ h}^{-1}$) alter the conclusion of the present study that rates of biomass loss cannot account for metabolism through metamorphosis. This is based on the following calculation: the cumulative oxygen consumption over a 10-day period (to stage iv) would be 19.2 nmol O_2 (at $84 \text{ pmol oxygen larva}^{-1} \text{ h}^{-1}$), equivalent to $0.30 \mu\text{g}$ of biomass (conversion of oxygen to biomass: Gnaiger, 1983). With a starting mean oocyte

biomass of $1.36 \mu\text{g}$ (Fig. 2, four cultures), the theoretical mass of a stage iv plantigrade would be $1.06 \mu\text{g}$, given a biomass loss to fuel metabolism of $0.30 \mu\text{g}$. A value of $1.06 \mu\text{g}$ is still less than any of the biomasses measured for plantigrades from all cultures.

Meeting the increased metabolic costs: roles of DOM and endogenous reserves

DOM in seawater may contribute significantly to the energy and growth requirements of marine invertebrate embryos and larvae (abalone: Jaeckle and Manahan, 1989a; sea urchins: Shilling and Manahan, 1990; Shilling and Bosch, 1994). In the present study, newly settled larvae (plantigrades), maintained in continuously flowing seawater, gained or did not change in biomass prior to their being able to feed on particles. This is in contrast to other studies with newly settled molluscs, raised in batch cultures, where loss of endogenous reserves has been observed (e.g., Rodriguez *et al.*, 1990). Our results indicate that both veligers and settled plantigrades of *H. rufescens* may meet most of their energy requirements through the uptake and metabolism of an exogenous food source, such as DOM. We calculated that plantigrades used their endogenous reserves to fuel only 39% of the metabolic cost of metamorphosis (days 6–9, Culture C, Table II). An exogenous source of energy has to be used to meet the remaining 61% of the energy costs. Upon metamorphosis, the maximum capacity for alanine transport (J_{max}) increased 3-fold (Fig. 3A, B) from stage ii to iv, with the sites of uptake being the velum (Dimster-Denk

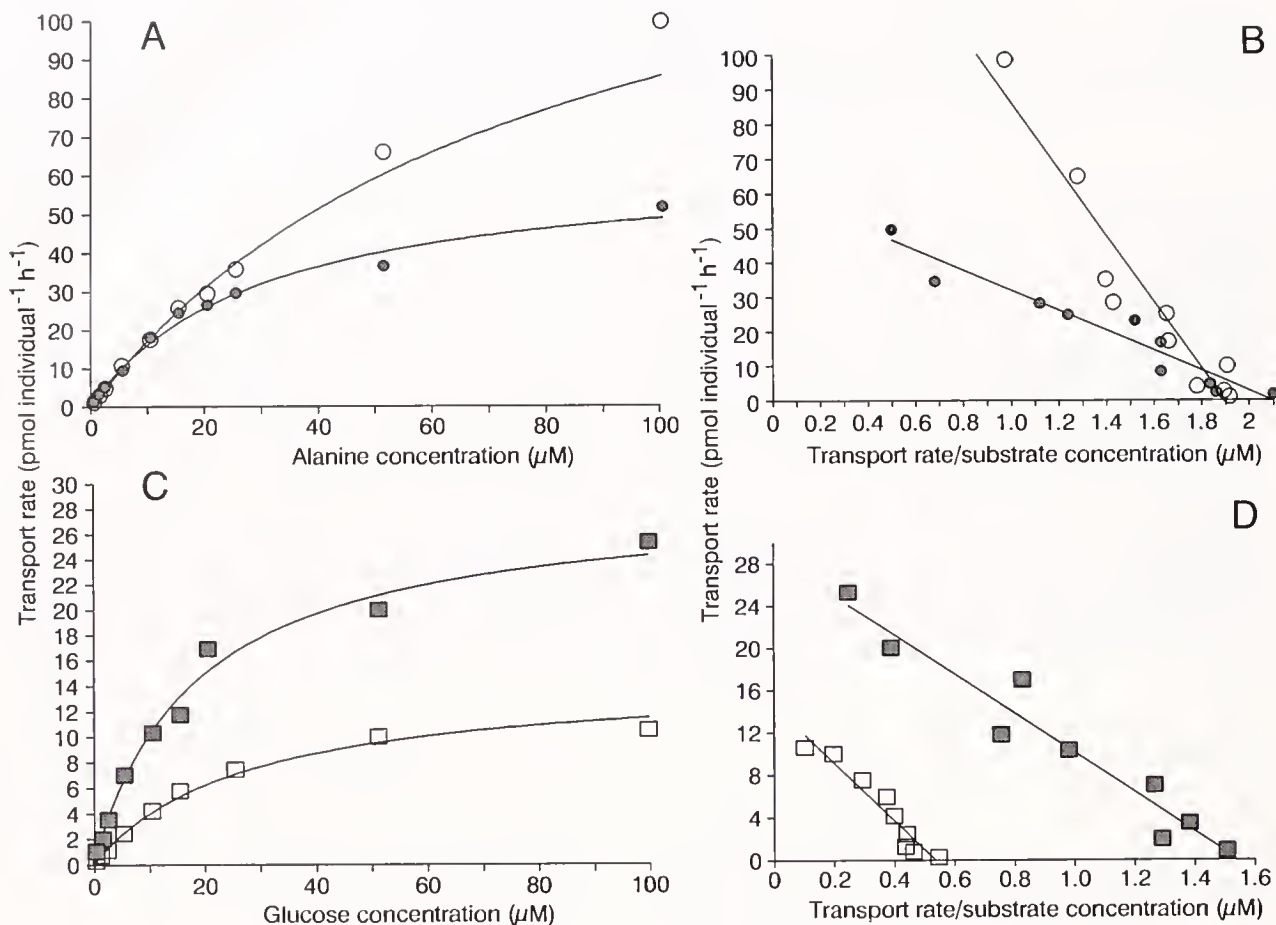


Figure 3. Kinetics of alanine (A & B) and glucose (C & D) transport by competent veligers (stage ii, closed symbols) and settled plantigrades (stage iv, open symbols). Each symbol represents a single uptake measurement; the curves were calculated using the K_i and J_{max} for each stage. The K_i and J_{max} values were as follows: (stage ii) alanine, 29 μM , 61.2 pmol alanine larva⁻¹ h⁻¹; glucose, 19 μM , 28.7 pmol glucose larva⁻¹ h⁻¹; (stage iv) alanine, 96 μM , 182.0 pmol alanine larva⁻¹ h⁻¹; glucose, 27 μM , 14.5 pmol glucose larva⁻¹ h⁻¹. (B) and (D) are Eadie-Hofstee plots of the data presented in (A) and (C), respectively.

and Manahan, unpub.) and presumably the developing gill buds in *H. rufescens* (cf. bivalve metamorphosis: Manahan and Crisp, 1983). The change in alanine transport kinetics is probably due to an increase in the number of transporter sites (indicated by increase in J_{max}) and a transition from localization of transport in the velum (once it is lost) to transport by other organs, such as the developing gill. Not all transport systems increase in capacity; the J_{max} for glucose transport decreased by half during metamorphosis of a swimming larva (stage ii) to a plantigrade (stage iv). However, if glucose transporters are present in the gill tissue, as is the case for adult mussels (Wright, 1988), then maximum transport capacity for sugars may increase as this tissue develops further.

The total oxygen consumed from oocyte to juvenile stage in 10 days of development was equivalent to 1.73 μg of biomass, more than the starting material in the

oocyte (Culture C: Table I, Fig. 2). Could the transport of DOM from seawater supply an amount of energy to lecithotrophic development equal to the initial maternal investment of energy in the oocyte? An estimate of the input of DOM can be obtained as follows. Based on maximum transport capacities (Fig. 3), glucose would be transported at a rate of 28 pmol h⁻¹ for a larva, decreasing to 14 pmol h⁻¹ for a juvenile. Over the 10-day period of development for which we have measurements of metabolic rates, these transport rates would provide 1.1 μg of organic material as glucose (8-day larval period at a rate of 28 pmol larva⁻¹ h⁻¹ = 968 ng glucose; 2-day juvenile period at a rate of 14 pmol larva⁻¹ h⁻¹ = 121 ng glucose). Similarly, during this 10-day period, alanine transport would yield 1.8 μg of material (8-day larval period at a transport rate of 61 pmol larva⁻¹ h⁻¹ = 1042 ng alanine; 2-day juvenile period at a transport rate of

182 pmol juvenile⁻¹ h⁻¹ = 777 ng alanine). Thus at maximum transport capacities, the combined input of alanine and glucose over the 10 days of development would be 2.9 μ g (1.8 μ g alanine plus 1.1 μ g glucose). This is more material than is required to supply the 1.73 μ g of biomass calculated to meet metabolic costs (Culture C, Table 1). Although unknown at this time, the substrate concentrations of dissolved amino acids and sugars in the pelagic (larva) and benthic (juvenile) environments of these animals are unlikely to be high enough for the transporters to reach $J_{\max}$ (concentrations of ca. 100 μ M would be needed, see Fig. 3B). Nonetheless, even if the amino acid and glucose transporters were to operate through development at only 20% of their maximum capacity, this would result in transport from the environment of about one-third of the 1.73 μ g of organic material required to supply metabolism (2.9 μ g $\times$ 0.2 = 0.58 μ g; 0.58/1.73 = 0.33). For transport rates to reach 20% of $J_{\max}$ in veliger larvae, amino acids would have to be at a concentration of 7 μ M and sugars at 5 μ M—the high end of reported concentrations, even in near-sediment waters (Williams, 1975). The metabolic requirement for high substrate concentrations in larvae of *H. rufescens* is largely set by the measured high K_t value of 29 μ M. This value is consistent with previous studies in which a K_t value of 23 μ M for alanine transport was reported for 2-day-old veligers of *H. rufescens* (Manahan *et al.*, 1989). Relative to the K_t values for amino acid transport reported for other species of marine invertebrate larvae (Manahan, 1990), veligers of *H. rufescens* have lower-affinity transporters (*i.e.*, higher K_t values). It is noteworthy that if abalone veligers had K_t values in the low micromolar range (similar to those reported for sea urchin larvae; Manahan *et al.*, 1989), an amino acid concentration of only 0.25 μ M would be required to have a transport rate of 20% of $J_{\max}$ in *H. rufescens*. Further study is required to understand the physiological significance of the range of K_t values found in different larval species and at different stages of development. Also, dissolved free amino acids and monomeric sugars in seawater make up only a small proportion of the total pool of DOM (Williams, 1975). The contribution that other components of the DOM pool might make to these animals is unknown, but their transport from seawater would only increase the total contribution of DOM to the energetics of development.

Metabolic rates increased 3- to 5-fold during metamorphosis (Table 1), while $J_{\max}$ for alanine increased 3-fold (Fig. 3). This phenomenon of up-regulation of maximum transport capacity for amino acids as metabolic demand increases during metamorphosis is consistent with other studies of animal development (mammals: Ferraris and Diamond, 1989; marine invertebrates: Manahan *et al.*, 1989). As the settled larva metamorphoses it

must supply the increased metabolic costs. An increased capacity to transport dissolved organic nutrients would provide a mechanism for meeting the higher costs of metamorphosis. Larvae undergoing metamorphosis have sufficient energy reserves to supply these metabolic needs but, if available, exogenous sources will be used in preference—resulting in individuals of greater biomass that are better able to survive further stresses (*e.g.*, delayed feeding) and potentially have higher survival rates as juveniles.

Acknowledgments

We thank John McMullen, Mike Machuzak, and the other staff of the AbLab (Port Hueneme, CA) for their indispensable help in conducting this project, Dr. William B. Jaecle for his advice in the conception of the project and for comments on the manuscript, and two anonymous reviewers for their comments on the manuscript. This work was supported by grants from the Office of Naval Research (N00014-90-J-1740) and by NOAA, Office of Sea Grant (U.S.C. Sea Grant) to D. T. Manahan.

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Effects of Hypoxia and Low-Frequency Agitation on Byssogenesis in the Freshwater Mussel *Dreissena polymorpha* (Pallas)

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Abstract. The effect of variations in PO_2 and agitation rate on byssogenesis, motility, and survival of the zebra mussel (*Dreissena polymorpha*) was investigated. Mussels exposed to a $PO_2 \leq 15.4$ torr exhibited increased mortality, reduced motility, and significant suppression of byssogenesis. At 7.7 and 15.4 torr, mean survival times were 5.2 and 5.8 days, maximum survival times being 15 and 16 days, respectively. After 21 days at a PO_2 of 23.1 torr, sample mortality was 33.3% and declined to 18.2% at 30.9 torr. There was no mortality at full air O_2 saturation (~ 154.3 torr). Adult zebra mussels exhibited the highest rate of byssogenesis in still water (0 cycles per minute [CPM]). Rate of byssogenesis progressively decreased as agitation rate increased. At 30 and 40 CPM, rate of byssal thread production was significantly lower than at 0 CPM. After 21 days, means of 58.6 and 44.8 byssal threads/mussel were found in the byssal mass of specimens exposed to 30 and 40 CPM, respectively, significantly fewer than the mean of 92.7 threads/mussel recorded in still water. Suppression of byssogenesis in *D. polymorpha* under hypoxic conditions is a response similar to that reported for the marine mytilid *Mytilus edulis*; however, suppression of byssogenesis with elevated agitation rate is the opposite response to that reported for *M. edulis*.

Introduction

The zebra mussel, *Dreissena polymorpha* (Pallas 1771), a freshwater bivalve mollusc, uses a byssus to secure to hard surfaces (Morton, 1969). The combination of a highly dispersive larval stage and retention of the byssal holdfast as an adult has made the zebra mussel a

damaging macrofouling pest species in North America and Europe (Mackie *et al.*, 1989; Claudi and Mackie, 1993).

Several environmental factors influence the rate of byssogenesis in marine byssate mussels such as *Mytilus edulis* L., *Geukensia demissus* (Dillwyn), and *Perna indica* Kariakose and Nair. These include temperature (van Winkle, 1970), current velocity (Lee *et al.*, 1989), ambient oxygen concentration (Ravera, 1950; Reish and Ayers, 1968), agitation rate (van Winkle, 1970; Young, 1985), salinity (Young, 1985; Mathew and Fernandez, 1992), and seasonal variations in abiotic factors (Price, 1980, 1982). Changes in a mussel's ability to produce byssal threads can have far-reaching consequences; wave action and tidal rhythms continually subject intertidal inhabitants to strong mechanical forces, and high levels of mortality occur when mussels become detached from the substratum (Harger and Landenberger, 1971).

There is wide taxonomic separation between mytilid and dreissinid bivalves. They also have different evolutionary histories, one evolving in high-energy intertidal marine habitats, the other in low-energy freshwater habitats. Their contrasting evolutionary histories may have resulted in differences in how the physiological processes that regulate the rate of byssogenesis respond to changing environmental conditions (Clarke and McMahon, 1996a).

The effects of current velocity and temperature on the rate of zebra mussel byssogenesis have been previously quantified and compared to the responses of marine mytilids (Clarke and McMahon, 1996b, c). In this paper we investigate the effects of hypoxia and low-frequency agitation on byssal thread production by *D. polymorpha* and compare the results to data published for mytilid species.

Materials and Methods

Specimen collection and maintenance

Specimens of *D. polymorpha* were collected from the upstream guide wall of Black Rock Lock, on the Niagara River, Buffalo, New York. They were transported overnight to The University of Texas at Arlington and maintained in aerated, dechlorinated tap water in a 280-l "Living Stream" holding tank at 5°C, without feeding, until used in experiments. Specimens have been maintained under these conditions with little mortality or loss of tissue mass for over 1 year (Chase and McMahon, 1994). Specimens of *D. bugensis* in the sample were visually identified according to descriptions by May and Marsden (1992) and removed. All specimens were used within 60 days of collection.

Responses to hypoxia

Prior to experimentation, mussels were acclimated in 17 l of aerated dechlorinated water to a test temperature of 25°C for 2 weeks. During acclimation, constant water temperature ($\pm 1^\circ\text{C}$) was maintained by holding aquaria in incubators. After acclimation, the byssus of experimental mussels was severed at the byssal gape with a razor blade. Immediately after byssus removal, mussels were placed on clear plastic plates ($14 \times 14 \times 0.2$ cm) located in the bottom of 17-l aquaria. Mussels were allowed to byssally reattach to the plastic plates for 12 h. The range of byssal thread production by individuals during the 12-h reattachment period was 4 to 26 threads/mussel.

Following the reattachment period, each plastic plate, together with either five or six attached mussels, was submerged in 4 l of dechlorinated tap water held in individual 5-l capped plastic chambers ($22 \times 22 \times 12$ cm) leaving 1 l of gas head-space. Water in these chambers was continuously bubbled with mixtures of oxygen and nitrogen gas. A Cameron GF-3 gas-mixing flowmeter regulated the gas mixture to maintain median dissolved oxygen partial pressures (PO_2) of 7.7 torr (5% air O_2 saturation at 25°C), 15.4 torr (10% air O_2 saturation), 23.1 torr (15% air O_2 saturation), 30.9 torr (20% air O_2 saturation) or 154.3 torr (100% air O_2 saturation). Media PO_2 was measured daily with a polarographic silver-platinum oxygen electrode (YSI Model 53). Chamber media were replaced every 2 days. The PO_2 of fresh medium was equilibrated to the experimental levels by perfusion with the appropriate gas mixture for 24 h before medium replacement.

Six chambers were used for each PO_2 treatment level, and the plastic plates were rotated among chambers daily. This rotation of specimens through the experimental chambers exposed individual mussels to each cham-

ber in the rotation for an equivalent time period. Consequently, each mussel was exposed equally to any "tank effect" that may have been associated with a particular chamber in the rotation. This allowed each individual mussel to be treated as an independent, or "true," replicate in the statistical analysis.

Mean shell length (SL)—the greatest linear dimension from the tip of the umbos to the posterior margin of the shell—of the mussels used for each treatment was 17.8 mm (± 3.2 SD, $n = 28$) at $PO_2 = 7.7$ torr; 15.4 mm (± 2.7 , $n = 28$) at 15.4 torr; 15.3 mm (± 2.4 , $n = 28$) at 23.1 torr; 17.4 mm (± 2.9 , $n = 28$) at 30.9 torr and 16.6 mm (± 2.6 , $n = 48$) at 154.3 torr.

The cumulative number of new byssal threads produced by reattaching mussels was recorded daily for 21 days after the initial reattachment (day 0). New byssal threads were counted by viewing the underside of the mussel, through the clear plastic plate, on a dissection microscope at 45 $\times$. Sites of thread attachment to the plate (*i.e.*, the plaques) were clearly visible when viewed in this manner. The location of new plaques was marked each day on the underside of the plate with a fine-tipped, waterproof, permanent ink marker. This procedure allowed quantitative determination of the effects of different PO_2 levels on byssal thread production. Time to mortality of individual mussels in the samples was recorded daily, as was time of any relocation of mussels on the plastic plates (detachment from the byssus followed by relocation and reattachment is common behavior among adults of this species, Mackie *et al.*, 1989).

The resulting data were tested for statistical significance by analysis of covariance (ANCOVA) in which PO_2 level was the main treatment, days was a repeated measure, and mussel SL and number of byssal threads present at day 0 were covariates. The null hypothesis that variations in oxygen partial pressure had no effect on the rate of byssal thread production was tested. A least significant difference (LSD) test was employed to detect any significant differences in rates of byssal thread production between experimental PO_2 levels.

Responses to low-frequency agitation

Specimens of *D. polymorpha* were acclimated and their byssus removed as previously described. Immediately after removal of the byssus, mussels were placed on clear plastic plates ($7 \times 7 \times 0.2$ cm) and allowed to byssally reattach for 12 h. The number of byssal threads produced by individuals during this period was 2 to 23 threads/mussel.

The plastic plate and attached mussel were then secured with a stainless steel spring clip to a fixed horizontal stage, supported from above and submerged in 15 l of dechlorinated tap water in a 17-l plastic aquarium. The

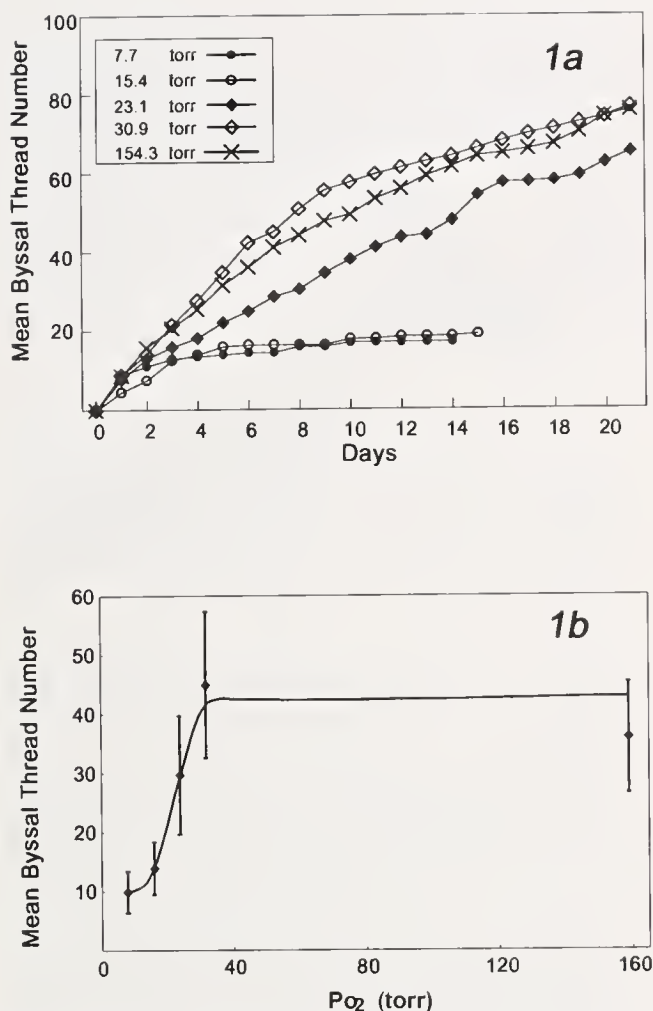


Figure 1. The effect of exposure to varying oxygen tensions on byssogenesis by specimens of the zebra mussel (*Dreissena polymorpha*) when exposed to PO_2 levels of 7.7, 15.4, 23.1, 30.9, and 154.3 torr. (a) Cumulative daily mean number of new byssal threads produced over a 21-day experimental period (or until 100% sample mortality). (b) Mean number of byssal threads ($\pm 95\%$ confidence limits) produced in a newly formed zebra mussel byssal complex following 7 days of exposure.

aquarium was fixed on the reciprocating plate of a New Brunswick Scientific Co. water bath shaker (model RW-650). A constant water temperature (25°C) was maintained in the aquaria by using the temperature control system of the water bath. This arrangement made it possible to keep the submerged horizontal stage and attached mussel stationary while the water-filled aquarium was reciprocally displaced over a horizontal distance of 4 cm. The fluid medium in the aquaria was maintained at a depth of 10 inches and reciprocally displaced at a rate of 0 CPM (cycles per minute), 10 CPM, 15 CPM, 30 CPM, or 40 CPM. Frictional forces caused by water movement agitated the stationary mussel with a two-phase alternating force. Exposure of individual mussels

was temporally separated to prevent pseudoreplication within treatments. The cumulative number of byssal threads produced by reattaching mussels exposed to varying agitation rates was recorded daily as previously described for specimens exposed to hypoxic conditions. Mussels were fed each day with rehydrated, washed, freeze-dried green algae, *Chlorella* sp., in recommended quantities (0.0032 g/mussel/day, Nichols, 1993). Medium was replaced every 2 days.

Before completion of the 21-day experimental period, some mussels spontaneously released from the byssus or were dislodged from their byssal hold-fasts by frictional forces caused by water movements. These individuals were not included in the calculation of mean daily byssal thread production. Rates of byssal thread production were calculated for mussels with mean SL of 21.9 mm (± 4.1 SD, $n = 20$) at 0 CPM; 17.0 mm (± 2.1 , $n = 20$) at 10 CPM; 17.2 mm (± 2.4 , $n = 20$) at 15 CPM; 16.3 mm (± 3.0 , $n = 20$) at 30 CPM; and 16.4 mm (± 3.1 , $n = 10$) at 40 CPM.

Results were analyzed using a repeated measures ANCOVA. Agitation rate was the main treatment effect; mussel SL and number of byssal threads present at day 0 were covariates. The null hypothesis was that variations in agitation rate had no effect on the rate of byssal thread production by *D. polymorpha*. Because mussel SL was entered into the analysis as a covariate, and because a test for homogeneity of this covariate showed no significant differences between treatment groups, variation in SL between treatments was not considered to be a confounding element in the statistical analysis.

Results

Response to hypoxia

Over the 21-day experimental period, specimens of *Dreissena polymorpha* produced byssal threads at a slower rate when exposed to low PO_2 levels of 7.7 and 15.4 torr than at higher levels of 23.1, 30.9, and 154.3 torr (Fig. 1a). Repeated measures ANCOVA indicated

Table 1

Repeated measures ANCOVA examining the effect of variations in partial pressure of oxygen (PO_2) and time (Days) on byssal thread production by *Dreissena polymorpha* over a 7-day exposure period at 25°C

Effect	df Effect	MS Effect	F	P
A) PO_2	4	7471	9.52	0.0001*
B) Days	6	5115	146	0.0001*
A $\times$ B Interaction	24	397	11.4	0.0001*

* Indicates a significant difference at P level shown.

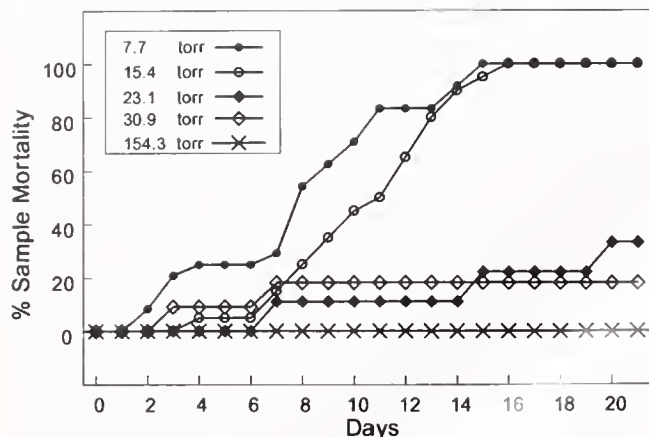


Figure 2. Daily mortality (as percentage of sample) of the zebra mussel (*Dreissena polymorpha*) when exposed to PO_2 levels of 7.7, 15.4, 23.1, 30.9, and 154.3 torr over a 21-day experimental period, or until 100% sample mortality occurred.

that PO_2 significantly influenced the rate of byssogenesis over the first 7 days of exposure (Table I). An LSD test indicated that the mean rate of byssogenesis was significantly greater ($P < 0.05$) for mussels exposed to high PO_2 (23.1, 30.9, and 154.3 torr) than for those exposed to low PO_2 (7.7 and 15.4 torr), over the first 7 days of exposure (Fig. 1b). The null hypothesis that oxygen concentration had no effect on the rate of byssal thread production was therefore rejected. As a result of differing production rates, specimens held at 23.1, 30.9, and 154.3 torr had significantly more new byssal threads in their byssal mass after 7 days exposure than those held at 7.7 or 15.4 torr (Fig. 1b). Mean byssal thread number is reported after the first 7 days only, because high mortality in samples

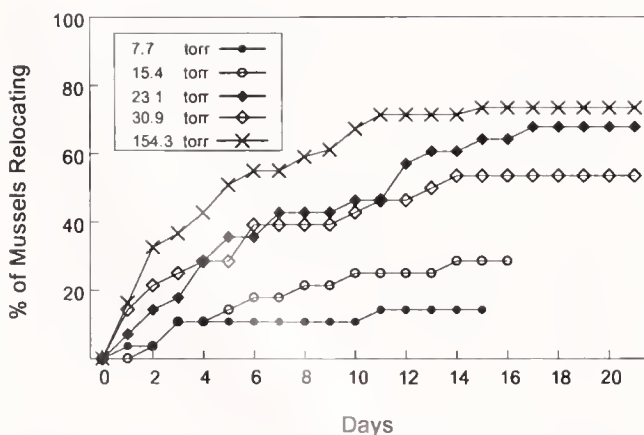


Figure 3. Percentage of the zebra mussel (*Dreissena polymorpha*) sample dropping from the byssal holdfast and relocating on the plastic plate, prior to death or the end of the 21-day experimental period (whichever came first), when exposed to PO_2 levels of 7.7, 15.4, 23.1, 30.9, and 154.3 torr.

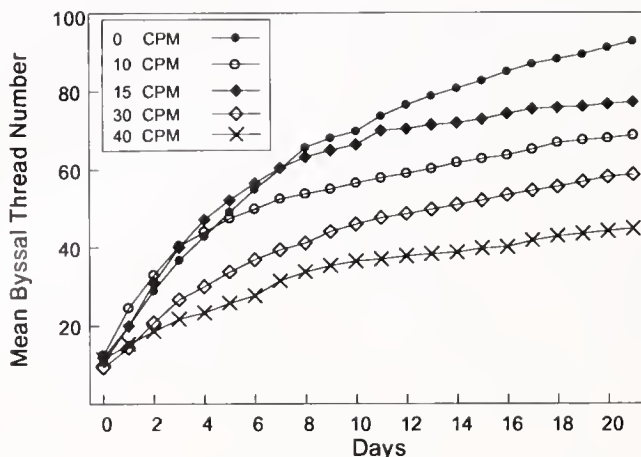


Figure 4. Cumulative daily mean number of byssal threads produced by specimens of the zebra mussel (*Dreissena polymorpha*) over a 21-day period, when exposed to agitation rates of 0, 10, 15, 30, and 40 cycles per minute (CPM).

exposed to 7.7 and 15.4 torr led to increasingly large disparities in sample sizes between treatment groups as time progressed.

There was very little new byssal thread formation by zebra mussels exposed to 7.7 or 15.4 torr over the maximum survival times of 15 and 16 days. However, typical byssal thread production curves, showing a steady increase in the number of threads produced with time, were recorded at a PO_2 of 23.1, 30.9, and 154.3 torr (Fig. 1a). For mussels exposed to 7.7 or 15.4 torr, mean survival times were 5.2 and 5.8 days, and maximum survival times were 15 and 16 days, respectively. At 23.1 torr, 33.3% sample mortality was observed by the end of the 21-day experimental period. Mortality declined to 18.2% at a PO_2 of 30.9 torr. No mortality was observed in the sample of mussels exposed at 154.3 torr (Fig. 2).

At 7.7 torr, 14.3% of the sample released from the byssus, relocated, and byssally reattached within the experimental chamber prior to death or the end of the experimental period (whichever came first). This figure was 28.6% at a PO_2 of 15.4 torr, 67.8% at 23.1 torr, 53.6% at 30.9 torr, and 73.4% at 154.3 torr (Fig. 3).

Response to agitation

Byssal thread production was inhibited by increasing agitation rates. Maximal byssal thread production over the 21-day exposure period was achieved at an agitation rate of 0 CPM, and minimum thread production occurred at 40 CPM (Fig. 4). Repeated measures ANCOVA indicated that agitation rate had a significant effect on byssal thread production rate over the 21-day test period. This resulted in rejection of our null hypothesis. Production rate varied significantly with the re-

Table II

Repeated measures ANCOVA examining the effect of variations in agitation rate (CPM) and time (Days) on byssal thread production by specimens of *Dreissena polymorpha* over a 21-day exposure at 25°C

Effect	df Effect	MS Effect	F	P
A) Agitation	4	31595	3.19	0.0173*
B) Days	20	16354	162	0.0001*
A × B Interaction	80	423	4.20	0.0001*

* Indicates a significant difference at *P* level shown.

peated measures factor of days, indicating that the rate of byssogenesis changes over time. Significant interaction between these two variables suggested that the pattern of change in byssogenesis rate over time is influenced by agitation rate (Table II).

An LSD test indicated that byssal thread production rates at low levels of agitation (*i.e.*, 0, 10, and 15 CPM) did not differ significantly from each other, nor did production rates at the higher agitation levels (*i.e.*, 30 and 40 CPM). All significant differences occurred between these two subsets of observations. Over the 21-day exposure period, mean byssal thread production rate was significantly suppressed at 40 CPM compared with that at 0, 10, and 15 CPM (Table III).

ANCOVA examination of the number of byssal threads found in the newly formed byssal mass of specimens after 21 days of exposure to varying agitation rates indicated that there was a significant effect of agitation rate ($F = 4.86$, $P < 0.001$). Significantly fewer threads were formed by mussels exposed to 40 CPM than by those exposed to 0, 10, and 15 CPM. Conversely, significantly more byssal threads were formed after 21 days by mussels exposed to 0 CPM than by those exposed to 10, 30, and 40 CPM (Fig. 5).

Discussion

Responses to hypoxia

The physiological processes regulating the rate of byssogenesis by *Dreissena polymorpha* appear to be hypoxia sensitive. Above 23.1 torr no suppression of byssogenesis was observed, but below 15.4 torr byssogenesis was significantly suppressed and sample mortality was 100%. A similar result has been reported by Reish and Ayers (1968) for the marine intertidal mytilid *M. edulis*. In this species, byssal thread production rates were normal at oxygen concentrations above 1 ppm (14.4 torr), but were inhibited by about 80% at $PO_2 \leq 1$ ppm, after 14 days of exposure.

A mean survival time of 82.8 h (range 34 to 156 h) has been reported for specimens of *D. polymorpha* accli-

mated to 25°C and exposed to acute hypoxic conditions (<3% of full air saturation, Matthews and McMahon, 1994). Survival times of only 24–48 h have been reported for mussels held at 23°–24°C under anoxic conditions (Mikheev, 1964). In our investigation, at low PO_2 levels mean survival time was 5 to 6 days, whereas at higher levels all or most of the sample survived the 21-day experimental period. Thus, although *D. polymorpha* appears to be capable of tolerating hypoxic conditions (≤ 15.4 torr) for longer periods than anoxia, there was very little difference in mean survival times at PO_2 levels ≤ 15.4 torr. The critical partial pressure of oxygen for both survival and byssogenesis of this species appears to lie somewhere between 15.4 and 23.1 torr.

The tendency for specimens of *D. polymorpha* to spontaneously release from the byssus and relocate to a new site declined with reduced oxygen concentration. While at elevated oxygen concentrations, mussels appeared to be very active. This suggests that motility and byssal thread secretion may be metabolically demanding activities for this predominantly sessile species. Such activities appear to be severely inhibited at oxygen concentrations below which efficient rates of aerobic metabolism cannot be maintained.

Responses to agitation

Increased levels of agitation resulted in a suppression of byssogenesis in the zebra mussel. This response is similar to that reported for the endobysate estuarine mussel *Geukensia demissa* (Dillwyn) (van Winkle, 1970). This marine mytilid responded to agitation rates that gradually increased from 0 to 86 CPM by progressively decreasing byssal thread production (van Winkle, 1970). However, when the intertidal epibyssate bivalve *M. edulis* was agitated once every 4.5 s (about equivalent to an agitation rate of 10 CPM in our study), specimens responded immediately by increasing the rate of byssal thread production to a level of twice that recorded when agitated only once every 27 s (Young, 1985). Thus, both

Table III

Least significant difference (LSD) range test for significant differences in mean rate of byssal thread production between specimens of *Dreissena polymorpha* exposed to agitation rates of 0, 10, 15, 30, and 40 cycles per minute (CPM) over a 21-day exposure period at 25°C

Agitation Rate (CPM)	10	15	30	40
0	0.079	0.455	0.001*	0.001*
10		0.297	0.112	0.017*
15			0.008*	0.001*
30				0.260

* Indicates a significant difference at *P* level shown.

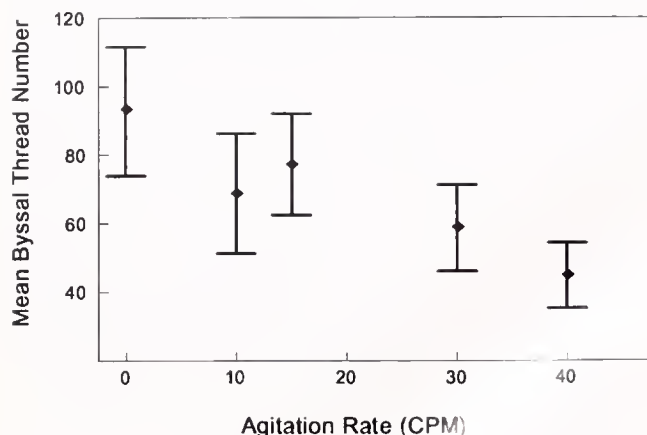


Figure 5. Mean number of byssal threads (±95% confidence limits) produced in a newly formed byssal complex of the zebra mussel (*Dreissena polymorpha*) following 21 days of exposure to agitation rates of 0, 10, 15, 30, and 40 cycles per minute (CPM).

D. polymorpha and *G. demissa* appear to respond differently than *M. edulis* to similar levels of elevated agitation.

These observations may possibly be explained by differences in the environments in which the three bivalve species have evolved. The zebra mussel is found in lotic and lentic systems in highest densities at depths greater than 3 m (Claudi and Mackie, 1993). Because the height of the vertical oscillation of waves is attenuated rapidly with depth (Wetzel, 1983), most zebra mussel populations probably reside below the depth of significant wave influence. Consequently, this species has not been exposed to the evolutionary pressures that would select for individuals that responded to wave agitation by increasing the rate of byssogenesis. This was demonstrated by our study, in which the highest level of byssal thread production was exhibited in still water. As levels of agitation progressively increased, mean rate of byssal thread production was suppressed. As a result, at 30 and 40 CPM the final number of threads found in the byssal mass was significantly reduced. This may explain why byssal thread production was inhibited in zebra mussels exposed to current velocities >0.2 m/s (Clarke and McMahon, 1996b)—the increased levels of agitation associated with high current velocity were responsible for the suppression.

Similarly, *Geukensia demissa* generally resides in estuarine mud flats, lying with most of its shell buried in the mud or gravel substratum and attaching byssal threads deeper in the sediment (Stanley, 1972). Consequently, like the zebra mussel, this species is not normally subjected to high levels of wave action or agitation.

In contrast to *D. polymorpha* and *G. demissa*, *M. edulis* inhabits a mechanically stressful, intertidal envi-

ronment, and has evolved to increase the strength of the byssal attachment in response to increases in agitation. A strong attachment is essential to the survival of this intertidal species if it is to withstand the forces imposed upon it by heavy wave action (Young, 1985). Field observations support this hypothesis. Populations of *M. edulis* in wave-exposed habitats had a mean attachment strength 15 times greater than those from wave-protected habitats (Witman and Suchanek, 1984). Similarly, byssal attachment strength in this species increased in correlation with strong wave action associated with increased Atlantic winds (Price, 1982). Studies indicate that both the quantity and quality of the byssal threads produced by *M. edulis* change in response to elevated levels of wave action; specimens from a surf-exposed shore produced 56% more byssal threads, that were on average 28% stronger (individual thread tensile strength), than those produced by mussels from a sheltered bay area (Moore, 1979).

It is unclear why increased agitation inhibits byssogenesis in zebra mussels. A crescent-shaped region (or distal depression) at the terminal end of the ventral groove of the foot is responsible for plaque formation in *D. polymorpha* (Rzepecki and Waite, 1993). The end of the ventral groove on the foot of *M. edulis* must remain firmly pressed against the substratum for about 1 minute while the plastic rodlet of the byssal thread and the attached plaque cure and become coherent (Mercer, 1952; Price, 1983). Continuously high levels of mechanical disturbance may thus interfere with the zebra mussel's ability to maintain contact between the distal depression of the foot and the substratum long enough for a cohesive byssal thread to form. It has also been suggested that agitation of the soft siphon tissues by the frictional forces caused by moving water and air bubbles prevents the zebra mussel from opening its shell valves (Kaster, 1995). Because the foot cannot be extended when the valves are closed, the pedal activity necessary for byssal thread production would be inhibited. Byssal thread formation may also be inhibited by low food availability (Clarke and McMahon, unpub. data). Consequently, agitation-induced valve closure could indirectly inhibit byssogenesis by limiting the mussel's food consumption and assimilation rates.

Conclusions

Differences in the byssogenic responses of *D. polymorpha* and *M. edulis* may result from specific adaptations to their respective habitats. Because *M. edulis* inhabits high energy intertidal ecosystems, it has evolved to increase byssal thread production and strengthen the byssal attachment in response to elevated agitation. In contrast, *D. polymorpha* is a sublittoral species adapted

to life in relatively stable, large bodies of fresh water, usually at depths below 3 m (Claudi and Mackie, 1993). The tendency for agitation to suppress byssogenesis in *D. polymorpha* suggests that this species would be unlikely to form functional byssal holdfasts in wave-influenced littoral regions. In fact, we observed, but did not quantify, a clear tendency for increased numbers of zebra mussels to spontaneously release from the byssus when exposed to high levels of agitation. Thus, mussels initially settling in wave-influenced littoral regions may detach from original attachment sites and move to deeper waters. Dispersal of shallow-settling (<1 m) juveniles to greater depths has been reported for *D. polymorpha* (Mackie *et al.*, 1989). *M. edulis* occurs in both marine intertidal and estuarine habitats (Seed, 1976). In estuaries, it can periodically be subject to hypoxic conditions. As an adaptation, *M. edulis* is capable of good regulation of oxygen uptake with progressive hypoxia and has a high tolerance for hypoxia (Bayne *et al.*, 1976). In contrast, zebra mussels are relatively intolerant of anoxia or hypoxia compared to the majority of other marine and freshwater bivalve species (Matthews and McMahon, 1994) and display little or no capacity to regulate rate of oxygen uptake (Alexander and McMahon, unpub. data). The poor hypoxia tolerance of *D. polymorpha* prevents its penetration into oxygen-depleted hypolimnetic waters (Matthews and McMahon, 1994) and is reflected by inhibition of byssogenesis at oxygen concentrations higher than those reported to inhibit byssogenesis in *M. edulis* (Reish and Ayers, 1968).

These differences among the byssogenic responses of *D. polymorpha* and *M. edulis* to environmental variables suggest that such responses are likely to be species-specific and cannot be generalized across different taxonomic groups.

Acknowledgments

Gary Dye, Lock Master at the U.S. Army Corps of Engineers Black Rock Lock, Buffalo, New York, provided experimental specimens. This *Dreissena polymorpha* research has been conducted as part of the Zebra Mussel Research Program of the U.S. Army Corps of Engineers under contract to the Center for Biological Macrofouling Research at the University of Texas at Arlington. Permission was granted by the Chief of Engineers to publish this information.

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Sulfide-Stimulation of Oxygen Consumption Rate and Cytochrome Reduction in Gills of the Estuarine Mussel *Geukensia demissa*

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Abstract. Organisms, such as the mussel *Geukensia demissa*, that inhabit high-sulfide sediments have mechanisms that impede sulfide poisoning of aerobic respiration. Oxygen consumption rates (nO_2) of excised ciliated gills from freshly collected *G. demissa* were stimulated 3-fold at sulfide concentrations between 200 and 500 μM and remained stimulated at 1000 μM . Maintenance of mussels in sulfide-free conditions resulted in less stimulation of gill nO_2 at <500 μM sulfide and inhibition between 500 and 1000 μM sulfide. Gills of *Mytilus galloprovincialis* from a sulfide-free environment were inhibited by $\geq 200 \mu M$ sulfide. These results indicate that sulfide stimulation of nO_2 may be correlated to environmental exposure to sulfide. Serotonin, a neurohormonal stimulant of ciliary beating, further increased sulfide-stimulated nO_2 , possibly in support of energy demand. Sulfide-stimulated nO_2 was negligible in boiled gills and was 61% inhibited by cyanide, implicating the participation of mitochondrial electron flux. Mitochondrial cytochromes *c* and oxidase oxidation/reduction state changed little at <500 μM sulfide, but reduction occurred at 500–2000 μM sulfide, suggesting that although cytochrome oxidation/reduction state may be regulated in the face of increased electron flux, regulation may fail at inhibitory sulfide levels. Sulfide-stimulated nO_2 may represent a detoxification mechanism in *G. demissa*.

Introduction

Hydrogen sulfide is common in marine reducing environments such as anoxic sediments, sewage sludge outfalls, deep-sea hydrothermal vents, and cold seeps. Because sulfide (the sum of H_2S , HS^- , and $S^{=}$ unless otherwise specified) is a potent inhibitor of mitochondrial cytochrome oxidase (Nicholls, 1975; Wilson and Erecinska, 1978; National Research Council, 1979) and can render hemoglobin nonfunctional through the formation of sulfhemoglobin (Berzofsky *et al.*, 1971; Carrico *et al.*, 1978), it is potentially toxic to a variety of aerobic organisms. In invertebrate/sulfur-oxidizing bacteria symbioses that inhabit reducing sediments and deep-sea hydrothermal vents, the host may exhibit mechanisms to reduce sulfide toxicity; these mechanisms include oxidation of sulfide to less toxic species and binding of sulfide to vascular and intracellular carrier proteins (reviewed in Somero *et al.*, 1989; Fisher, 1990; Childress and Fisher, 1992). Organisms without sulfur-oxidizing symbionts must also detoxify sulfide to survive in marine reducing environments. Catalysis of sulfide oxidation has been demonstrated in a variety of nonsymbiotic invertebrates and may involve mechanisms such as sulfide oxidases and nonenzyme catalysts such as metals and organic compounds (reviewed in Somero *et al.*, 1989).

An additional mechanism for sulfide detoxification may occur in mitochondria, which may harness the energy from sulfide oxidation. Sulfide oxidation coupled with ATP production has been demonstrated in mitochondria isolated from the symbiotic clam *Solemya reidi*, with thiosulfate as the major endproduct (Powell and Somero, 1986; O'Brien and Vetter, 1990), from the liver

Received 15 July 1996; accepted 16 September 1996.

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of a sulfide-tolerant fish (Bagarinao and Vetter, 1990), and from the symbiont-free burrowing polychaete *Heteromastus filiformis* (Oeschger and Vismann, 1994). It is not known if mitochondrial sulfide oxidation occurs in intact tissues, if the energy potentially gained from mitochondrial sulfide oxidation contributes significantly to cellular work demands (Doeller, 1995), or how widespread such a pathway may be among organisms that encounter sulfide.

In the present study, we investigated the interaction between sulfide and mitochondria in the intact excised gill of the estuarine ribbed mussel *Geukensia demissa* (formerly *Modiolus demissus*). These mussels are commonly found partially or totally buried in sediments of *Spartina* grass tide marshes (Kuenzler, 1961). Animals at the sediment surface may exhibit air-gaping behavior by keeping their valves open during low tide (Lent, 1968). Porewater around sediment associated with *Spartina* can contain high (millimolar) concentrations of sulfide (Carlson and Forrest, 1982; King *et al.*, 1982; this study), thus *G. demissa* is most likely subject to chronic sulfide exposure. The objectives of the present study were to characterize environmental sulfide concentrations where *G. demissa* was collected; to measure oxygen consumption rates in excised gills as a function of ambient sulfide exposure in the presence and absence of the neurohormone serotonin, which stimulates ciliary beating and hence ATP demand (Clemmesen and Jørgensen, 1987); and to determine the effects of sulfide and serotonin on mitochondrial cytochrome oxidation/reduction state in excised gills. For comparison, parallel experiments were conducted with excised gills from *Mytilus galloprovincialis* or *Mytilus edulis* collected from habitats in which sulfide concentrations are most likely negligible.

Materials and Methods

Mussel collection and maintenance

Geukensia demissa (60–70 mm shell length) was collected during low to mid tide from *Spartina* grass beds on Dauphin Island, Alabama. In the laboratory, the mussels were maintained in recirculating seawater aquaria at 23°C. Some aquaria were partially filled with sediment obtained from the collection site and periodically seeded with dried *Spartina* pieces to stimulate sulfide production. The *G. demissa* specimens in these mudtanks were partially buried in the sediment to maintain their exposure to sulfide-enriched conditions. Animals were maintained in artificial seawater (Tropic Marin) at 24–30 ppt to correspond with collection salinity. *Mytilus galloprovincialis* (60 mm in shell length) obtained from offshore oil platforms in the Santa Barbara Channel, California, and *M. edulis* (50–60 mm in shell length) ob-

tained from pilings of the Goleta Pier, California, were maintained for several weeks in recirculating seawater aquaria at 23°C and 30–35 ppt salinity prior to experiments. Experiments were performed at 20°C at maintenance salinity.

In situ sulfide measurements

The reaction of sulfide with 2,2'-dipyridyl disulfide (2-PDS) to form 2-thiopyridone, detected spectrophotometrically at 343 nm, is a simple and sensitive assay for determining hydrogen sulfide in aqueous solution (Svenson, 1980). Sulfide standards treated with 2-PDS were found to have stable absorbances for at least 6 days when kept at room temperature or in the refrigerator. Sulfide was not lost from standards subjected to our collection protocol (described below). Hemolymph of freshly collected *G. demissa* and 1 mM sodium azide added to prevent bacterial growth did not interfere with the detection of internal sulfide standards.

Sediment porewater was sampled in May 1995 at *G. demissa* collection sites. A 50-cm piece of 18-gauge stainless steel tubing with small perforations near the tip and a female locking Luer connection at the base was attached to a 10-ml plastic syringe and inserted several centimeters into the sediment where many of the buried *G. demissa* were found. Aliquots (1–10 ml) of porewater were collected into the syringe, then filtered through a 13-mm glass-fiber filter mounted in a Millipore swinnex syringe filter holder. After the first few drops of filtered porewater were discarded, 50–200- μ l aliquots were immediately pipetted into a 2.0-ml assay mixture containing 0.75 mM 2-PDS, 50 mM sodium phosphate, and 1 mM sodium azide (pH 6.5) for later determination of hydrogen sulfide. Internal fluid (a mixture of hemolymph and seawater contained in the mantle cavity) was obtained from specimens of *G. demissa* within minutes of their removal from the sediment by using a syringe with the needle inserted between the valves. Aliquots (250–500 μ l) of this fluid were pipetted into the assay mixture for determination of sulfide. The sulfide in these samples was quantified the following day in the laboratory. Sodium sulfide standards prepared and added to the assay mixture in the field were used to construct a standard curve.

Excised gill closed-chamber respirometry

Sample preparation. Intact demibranchs were excised and placed in dishes containing filtered (0.45 μ m) artificial seawater (FASW) for at least 1 h to allow mucus to clear from the gill surface. Gill pieces (0.1–0.2 g wet weight) were then placed on stainless steel screen holders (Doeller *et al.*, 1990) and inserted into a closed dual-chamber respirometer (Oroboros Oxygraph; Paar KG,

Graz, Austria) containing 6 ml stirred (500 rpm), air-equilibrated FASW at 20°C. After experiments, gill pieces were dried for at least 48 h at 70°C and weighed. Oxygen consumption rates are presented as mean $\mu\text{mol O}_2 \text{ s}^{-1} \text{ mg (dry weight)}^{-1} \pm$ standard deviation.

Effects of sulfide on gill oxygen consumption rate. Three determinations of normoxic oxygen consumption rate were made, each over roughly 5-min intervals. Oxygen concentration in the respirometer was maintained close to air-saturation by periodically adding oxygen-equilibrated FASW through the injection port with a microliter syringe (Hamilton). Gills were then exposed to sulfide by addition of microliter aliquots of freshly made 30 mM sodium sulfide in FASW. Measurements were taken for at least 5 min at sulfide concentrations of 10–2000 μM . For each sulfide concentration, the rate of oxygen consumption due to spontaneous oxidation of sulfide was determined using respirometer chambers without excised gills. These background rates, which were $\leq 10\%$ of the rates observed with freshly collected gills for sulfide concentrations of 500 μM or less, were subtracted from experimental oxygen consumption rates. Initial (stated) sulfide concentrations dropped less than 5% due to spontaneous sulfide oxidation during the course of a measurement. Oxygen consumption rates were measured within the $p\text{O}_2$ range of 156 torr to 19 torr and were found to be independent of $p\text{O}_2$ within this range (data not shown).

Treatments. The effects of sulfide on gill oxygen consumption rate were determined using groups of mussels as follows: *G. demissa* within 4 days from field collection (termed freshly collected), *G. demissa* maintained in aquaria with sulfide-enriched sediment for approximately 2 months (termed mudtank-maintained), *G. demissa* maintained in aquaria without sulfide sediment for about 2 months (termed sulfide-free), and *Mytilus galloprovincialis* or *M. edulis* with no history of sulfide exposure. This allowed an assessment of the effects of ambient sulfide exposure. To determine the relationship between energy demand and sulfide effects on oxygen consumption rate, the neurohormone serotonin (10 μM) was used to stimulate ciliary beating (Clemmesen and Jørgensen, 1987). The cytochrome oxidase inhibitor cyanide was used to determine mitochondrial participation in sulfide-mediated oxygen consumption. Sulfide-mediated oxygen consumption rate of gill pieces boiled for 10 min was measured to determine the proportion that is protein-based.

Excised gill optical spectrophotometry

Sample preparation. Excised intact demibranchs were kept in FASW for at least 1 h. Demibranch pieces, about 1 cm^2 , were mounted between two frames, one made

from stainless steel screen (60 mesh; outer dimension 15 $\times$ 15 mm; inner dimension 8 $\times$ 6 mm) and one from black plastic (outer dimension 14 $\times$ 12 mm; inner dimension 7 $\times$ 4 mm). Mounted gills were placed in a water-jacketed (20°C) cuvette containing 6 ml FASW buffered with 25 mM Tris (pH = 8.0) below a 6-ml gas space. A sulfide-insensitive polarographic oxygen sensor (Orbisphere model 2120, Geneva, Switzerland) as the floor of the cuvette was used to continuously measure solution $p\text{O}_2$. A stopper that sealed the top of the cuvette contained a stirring motor and stainless steel turbine (460 rpm), inflow and outflow gas lines, and an injection port. Optical spectra were acquired with a recording spectrophotometer equipped with a scattered transmission accessory and a digital data acquisition and analysis system (Cary model 14; Aviv Associates, Lakewood, NJ). Optical spectra were recorded from 400 to 650 nm at 1-nm intervals. Effects of treatments on oxidation/reduction state of mitochondrial cytochromes were determined from difference spectra of gills under treatment conditions minus the same gills under air-saturated seawater conditions. Spectral features associated with cytochrome reduction observed in these difference spectra were quantified by determining optical density differences (ΔOD s) between absorption maxima and a baseline obtained by visually fitting data points adjacent to peaks to a straight line. Absorption maxima observed at 550 nm were ascribed to cytochrome *c*. Absorption maxima observed at 605 nm in *G. demissa* gills and at 610 nm in *M. edulis* gills were ascribed to cytochrome oxidase. Percent reduction of cytochromes was calculated by comparing treatment ΔOD to ΔOD taken from difference spectra of gills exposed to conditions under which mitochondrial cytochromes are maximally reduced (addition of 1 mg ml^{-1} sodium dithionite to the medium) minus gills under air-saturated conditions.

Effects of sulfide on mitochondrial cytochrome oxidation/reduction state. Optical spectra of air-equilibrated gills were acquired while passing humidified air through the spectrophotometer cuvette at a flow rate of 100 ml min^{-1} , regulated by a mass flow controller (Tylan, Carson, CA). Gills were then exposed to increasing concentrations of sulfide (20–2000 μM) by adding microliter aliquots of freshly made 30 mM sodium sulfide solution through the injection port of the cuvette with a Hamilton syringe. Based on a pK of 6.59 for the dissociation of sulfide in 35 ppt seawater at 20°C (Millero *et al.*, 1988) and a solubility of 146 $\mu\text{M H}_2\text{S torr}^{-1}$ (Millero, 1986), a sulfide concentration of 20–2000 μM at pH 8.0 corresponds to a $p\text{H}_2\text{S}$ of 0.005–0.51 torr. To prevent loss of H_2S from the medium, hydrogen sulfide gas was metered into the humidified air by using a syringe pump (Harvard) to match $p\text{H}_2\text{S}$ with sulfide concentration. Oxygen partial pressure was found to stabilize within 10 to 15

min after exposure to each sulfide concentration, at which time optical spectra were acquired.

Treatments. Groups of mussels similar to those used in closed-chamber respirometric experiments were used in spectrophotometric experiments: freshly collected *G. demissa*, sulfide-free *G. demissa*, and *M. edulis* collected from a sulfide-free environment and maintained without sulfide. The neurohormone serotonin (10 μ M) was used to determine the relationship between energy demand and cytochrome oxidation/reduction state with and without sulfide.

Results

In situ hydrogen sulfide measurements

Sediment porewater was collected at several intertidal sites within the *Spartina* marsh where *Geukensia demissa* seemed abundant. All sites were among *S. alterniflora*. At sampling time during midtide, sediments were either exposed to air or covered by 20–40 cm of overlying seawater. Sulfide concentration in porewater samples ranged from 1.3 mM to 8.1 mM, with a mean value of 4.8 mM ($n = 6$). Fluid consisting of a mixture of hemolymph and seawater contained within the mantle cavity of 10 mussels (≈ 60 –70 cm shell length) collected from a clump of *S. alterniflora* roots had neither a sulfide odor nor a detectable sulfide concentration. The sulfide concentration in the porewater sample collected nearest to these mussels was 1.3 mM. The absence of detectable sulfide in the mantle cavity fluid may have resulted from tissue sulfide oxidation or from exchange of mantle cavity fluid with overlying seawater possibly brought on by the mechanics of dislodging the animal during collection.

Sulfide stimulation of oxygen consumption rate in gills of freshly collected *Geukensia demissa*

In the absence of sulfide, *G. demissa* gills exhibited a mean normoxic oxygen consumption rate (nO_2) of 15.0 ± 2.8 pmol O_2 s⁻¹ mg⁻¹ ($n = 6$; Fig. 1A). The addition of sulfide stimulated nO_2 to a maximum near 50 pmol O_2 s⁻¹ mg⁻¹ ($n = 6$) at 200–500 μ M sulfide (Fig. 1A). At 1000 μ M sulfide, nO_2 averaged 37.3 ± 7.6 ($n = 6$; Fig. 1A).

Addition of 10 μ M serotonin in the absence of sulfide resulted in a 1.8-fold stimulation of nO_2 to 27.5 ± 5.6 ($n = 5$; Fig. 1A). As before, sulfide stimulated nO_2 , which reached a maximum of 78.1 ± 12.0 pmol O_2 s⁻¹ mg⁻¹ ($n = 5$) at 200 μ M sulfide (Fig. 1A). At 1000 μ M sulfide, nO_2 remained elevated at 55.9 ± 10.8 ($n = 5$; Fig. 1A).

Sulfide-stimulated oxygen consumption rate in gills of mudtank-maintained *Geukensia demissa*

Gills of mudtank-maintained *G. demissa* exhibited a mean nO_2 of 10.8 ± 2.5 pmol O_2 s⁻¹ mg⁻¹ ($n = 10$; Fig.

1A). At 200 μ M sulfide, sulfide-stimulated nO_2 was 55.3 ± 9.4 pmol O_2 s⁻¹ mg⁻¹ ($n = 5$), comparable to the rate exhibited by gills of freshly collected *G. demissa* at 200 μ M sulfide (Fig. 1A). Thus the sulfide-stimulated nO_2 at 200 μ M sulfide does not decline over time, unlike the rate observed in animals kept in the absence of sulfide for a similar time period (see below). This result indicates that animals kept in aquaria with sulfide-enriched sediment maintain a capacity for sulfide oxidation similar to that of freshly collected animals.

Effects of boiling on sulfide-stimulated oxygen consumption rate by gills of mudtank-maintained *Geukensia demissa*

Geukensia demissa gills boiled for 10 min exhibited negligible rates of sulfide-stimulated oxygen consumption over the entire range of sulfide concentrations (50–1000 μ M; Fig. 1A).

Sulfide-stimulated oxygen consumption rate in gills of *Geukensia demissa* maintained without sulfide for about 2 months

Strikingly different responses to sulfide were observed between gills of freshly collected mussels and gills of mussels maintained in aquaria without sulfide sediment. Mean nO_2 of gills from sulfide-free *G. demissa* in the absence of sulfide was 12.7 ± 3.2 pmol O_2 s⁻¹ mg⁻¹ ($n = 12$; Fig. 1B), similar to that of gills from freshly collected mussels. Maximum sulfide-stimulated nO_2 exhibited at 200 μ M sulfide, was 30.0 ± 4.9 pmol O_2 s⁻¹ mg⁻¹ ($n = 6$; Fig. 1B), 60% of that exhibited by gills of freshly collected mussels. At greater than 200 μ M sulfide, nO_2 noticeably declined, and at 1000 μ M sulfide they were no different than control ($P = 0.27$; ANOVA; Fig. 1B).

Treatment with 10 μ M serotonin resulted in a 2.0-fold stimulation of nO_2 to 27.5 ± 5.6 pmol O_2 s⁻¹ mg⁻¹ ($n = 7$; Fig. 1B). Maximum sulfide-stimulated nO_2 , observed at 100 μ M sulfide, was 45.1 ± 10.1 pmol O_2 s⁻¹ mg⁻¹ ($n = 7$; Fig. 1B), 59% of that exhibited by gills of freshly collected mussels at 200 mM sulfide. At greater than 100 μ M sulfide, nO_2 declined dramatically, and at 1000 μ M sulfide they were 13.2 ± 5.7 pmol O_2 s⁻¹ mg⁻¹ ($n = 7$; Fig. 1B), 48% of the serotonin-stimulated control value ($P < 0.05$; ANOVA).

Sulfide-stimulated oxygen consumption rate in gills of *Mytilus galloprovincialis* collected from a sulfide-free habitat

The mean nO_2 exhibited by *M. galloprovincialis* gills in the absence of sulfide was 15.0 ± 2.3 pmol O_2 s⁻¹ mg⁻¹ ($n = 5$; Fig. 1C), similar to rates exhibited by *G. demissa* gills under the same conditions (Fig. 1A, B). Exposure

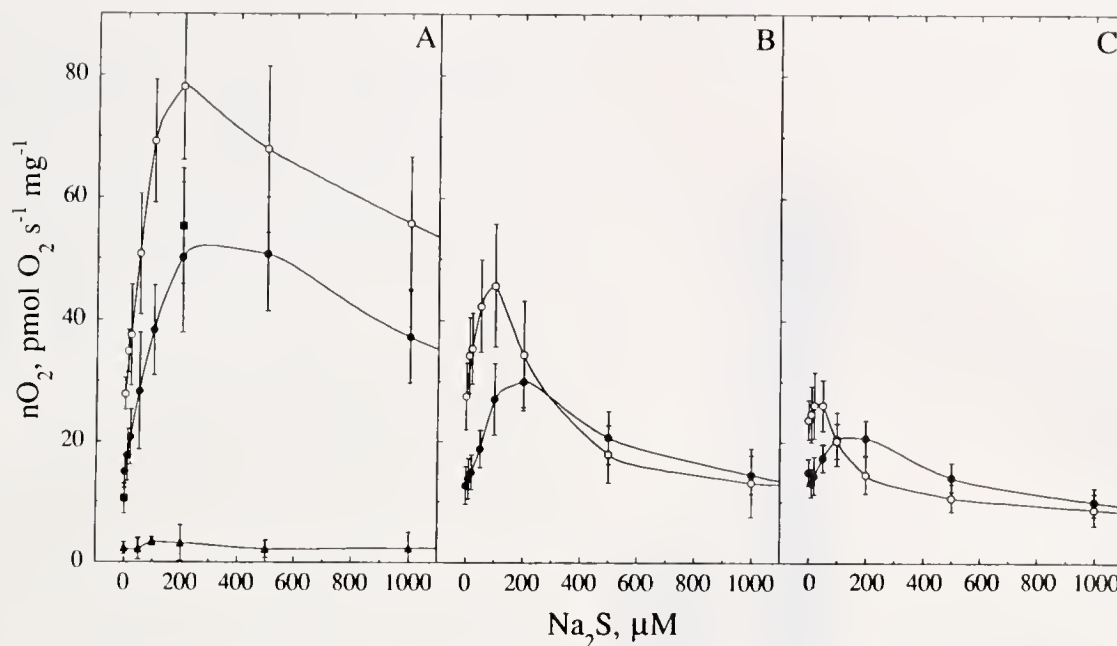


Figure 1. Oxygen consumption rate (nO_2) of excised mussel gills as a function of sulfide concentration, in the presence (open symbols) and absence (closed symbols) of serotonin. Rates are given as mean $\pm$ SD. (A) Gills of freshly collected *Geukensia demissa* (circles); gills of mudtank-maintained *G. demissa* (squares); boiled *G. demissa* gills (diamonds). (B) Gills of sulfide-free *G. demissa* (C) Gills of *Mytilus galloprovincialis*.

to sulfide slightly but significantly increased nO_2 , with a maximum near 20 $\text{pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ ($n = 5$) observed at 100–200 μM sulfide ($P < 0.05$ for both treatments; ANOVA; Fig. 1C). At 1000 μM sulfide, nO_2 was $10.1 \pm 2.4 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ ($n = 5$; Fig. 1C), 67% of the control value.

M. galloprovincialis gills exposed to 10 μM serotonin in the absence of sulfide exhibited a 1.6-fold stimulation of nO_2 to $23.7 \pm 3.3 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ ($n = 5$; Fig. 1C). Oxygen consumption rates were significantly inhibited by $\geq 100 \mu\text{M}$ sulfide ($P < 0.05$ for 100 μM and 200 μM sulfide; ANOVA), and at 1000 μM sulfide were $8.9 \pm 2.6 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ ($n = 5$; Fig. 1C), 38% of the serotonin-stimulated control value.

Effect of cyanide on sulfide-stimulated oxygen consumption rate by gills of mudtank-maintained *Geukensia demissa*

To test for mitochondrial participation in sulfide-stimulated nO_2 , the effect of cyanide, an inhibitor of mitochondrial cytochrome oxidase, was determined in gills of mudtank-maintained *G. demissa* (Fig. 2). Control nO_2 of these gills (see Fig. 1A) was inhibited by more than 90% in the presence of 1 mM potassium cyanide (Fig. 2). Oxygen consumption rate in the presence of 200 mM sulfide (see Fig. 1A) was inhibited by cyanide to 21.6

$\pm 7.2 \text{ pmol O}_2 \text{ s}^{-1} \text{ mg}^{-1}$ ($n = 5$; Fig. 2), representing a 61% inhibition of the rate in the absence of cyanide.

Effects of sulfide on the oxidation/reduction state of cytochromes *c* and oxidase in mussel gills

Difference spectra of *G. demissa* gills exposed to sodium dithionite (1 mg ml^{-1}) minus the same gills equilibrated with air showed reduced minus oxidized mitochondrial cytochromes *b* and *c* and oxidase (Fig. 3, upper line). Under normoxic conditions, addition of sulfide at 20–200 μM did not alter gill mitochondrial cytochrome oxidation/reduction state, as indicated by difference spectra of gills in the presence of 50 μM sulfide minus the same gills in the absence of sulfide (Fig. 3, lower line). Steady-state nO_2 of gills in the spectrophotometer cuvette (data not shown) increased in response to sulfide in a manner similar to that of gills in the closed-chamber respirometer (Fig. 1); however, high sulfide concentrations (1000 μM) were required to cause cytochrome reduction (Fig. 3, middle line).

Reduction of cytochromes *c* and oxidase in *G. demissa* gills neared 50% at sulfide concentrations of 500 μM or greater (Figs. 4A, B; 5A, B), and neither cytochrome reached full reduction at sulfide concentrations up to 2000 μM . Results from gills of freshly collected and sulfide-free *G. demissa* were similar, although cytochrome

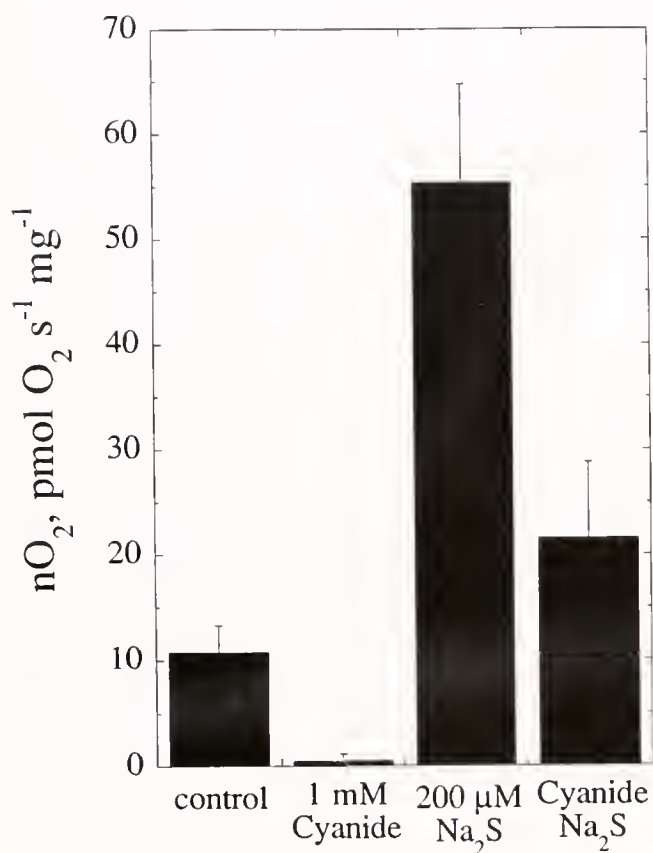


Figure 2. Oxygen consumption rate (nO_2) of excised gills of mud-tank-maintained *Geukensia demissa* in the presence of sulfide ($200\ \mu M$ Na_2S) and cyanide ($1\ mM$). Rates are given as mean $\pm$ SD.

c in gills of freshly collected *G. demissa* exhibited decreased reduction in the presence of serotonin (Fig. 4A), and the percentage reduction of cytochrome oxidase was slightly higher in gills of sulfide-free mussels at sulfide concentrations of $500\ \mu M$ or greater (Fig. 5B). Little reduction of cytochromes *c* and oxidase of *M. edulis* gills was observed below $500\ \mu M$ sulfide, and less than 100% reduction was observed at 500 – $2000\ \mu M$ sulfide (Figs. 4C, 5C); these results were similar to those obtained with *G. demissa* gills.

Addition of $10\ \mu M$ serotonin to *G. demissa* and *M. edulis* gills increased the steady-state rates of oxygen consumption (data not shown) in a manner similar to that exhibited by gills in the closed-chamber respirometer (Fig. 1) but produced little detectable change in cytochrome redox state (Figs. 4, 5). Changes in percentage reduction of cytochromes *c* and oxidase in response to sulfide were similar to results observed in the absence of serotonin (Figs. 4, 5).

Discussion

Animals that routinely encounter sulfide in their habitats possess mechanisms to oxidize sulfide to less toxic

forms, thus preventing sulfide poisoning of aerobic respiration (Somero *et al.*, 1989). We investigated two genera of mussels exposed to different environmental levels of sulfide to determine whether a correlation exists between environmental sulfide exposure, oxygen consumption rate, and cytochrome redox state. Ciliated gills, which exist at the interface between animal and environment, were used as models of aerobically active tissues that are sensitive to environmental conditions.

The oxygen consumption rates of excised gills of *Geukensia demissa*, *Mytilus galloprovincialis*, and *M. edulis* measured before and after stimulation with $10\ \mu M$ serotonin were similar to those previously reported for gills of *M. edulis* under similar conditions (Clemmesen and Jørgensen, 1987; Doeller *et al.*, 1993). However, sulfide effects on oxygen consumption rate of gill tissue appeared to be correlated with environmental sulfide exposure. Sulfide-stimulation of oxygen consumption rate was greatest in gills of *G. demissa* with a history of sulfide exposure—that is, in freshly collected mussels and mussels maintained in sulfide-enriched mudtanks. Gills of *G. demissa* exposed to sulfide-free conditions for more than 2 months exhibited substantially reduced rates of sulfide-stimulated oxygen consumption. Gills of *M. galloprovincialis*, a species that does not routinely encounter high sulfide levels, exhibited the lowest rates of sulfide-stimulated oxygen consumption. The reduced level of sulfide-stimulated oxygen consumption in animals not exposed to sulfide may reflect the inactivation or lack of a metabolic pathway involved in sulfide oxidation. The presence of a small amount of sulfide-stimulated oxygen consumption in *M. galloprovincialis* may be the result of a general tissue capacity for sulfide oxidation, as discussed by Anderson (1995) in reference to sulfide oxidation capacity in *M. californianus* gills, and may not reflect adaptation to environmental sulfide exposure.

Sulfide-stimulation of oxygen consumption rate may reflect oxidation of sulfide to less toxic forms such as thiosulfate, sulfite, sulfate, or elemental sulfur, and this process may function in part to protect aerobic metabolism from sulfide poisoning of cytochrome oxidase. Mitochondria isolated in the uncoupled state from *G. demissa* gills showed inhibition of oxygen consumption beginning at $5\ \mu M$ sulfide (Lee, unpub.), suggesting that if cytochrome oxidase is as sensitive in the intact tissue as it is in isolated uncoupled mitochondria, intracellular sulfide concentration must be maintained at a low level. The oxygen consumption results reported here are consistent with a protective function of sulfide oxidation.

The enhanced capacity for sulfide oxidation exhibited by *G. demissa* with a history of sulfide exposure may be due to non-enzyme-catalyzed sulfide oxidation, sulfide-oxidase enzymes, or mitochondrial oxidation of sulfide. Because boiled gills exhibited no sulfide-stimulated oxy-

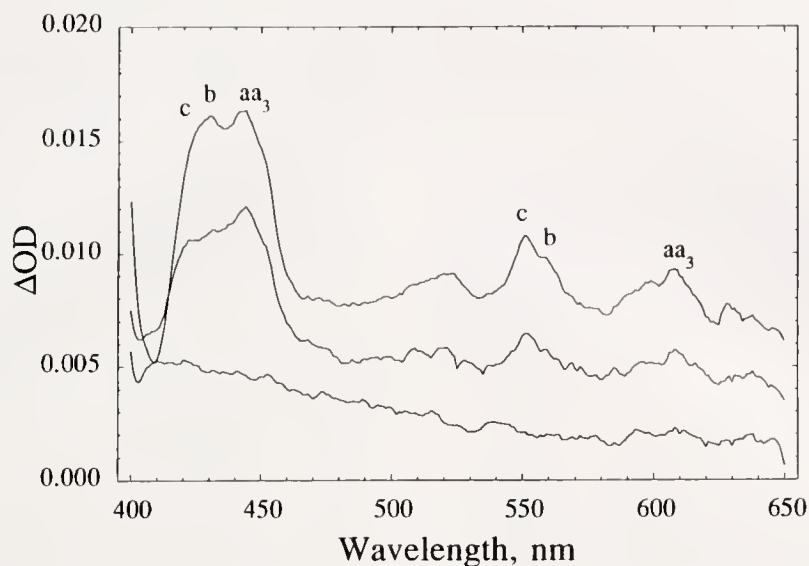


Figure 3. Difference spectra of gills of freshly collected *Geukensia demissa*: in seawater with 1 mg ml⁻¹ dithionite minus the same gills in air-equilibrated seawater (top spectrum); in seawater with 1000 μ M Na₂S minus the same gills in air-equilibrated seawater (middle spectrum); and in seawater with 50 μ M Na₂S minus the same gills in air-equilibrated seawater (bottom spectrum). The top difference spectrum shows the full complement of reduced minus oxidized mitochondrial cytochromes oxidase (*aa*₃), *b*, and *c*.

gen consumption, mechanisms responsible for sulfide oxidation may involve sulfide-oxidase enzymes or mitochondria since both are probably more sensitive to heat

denaturation than are sulfide-oxidizing catalysts such as metal ions or organic compounds (Chen and Morris, 1972).

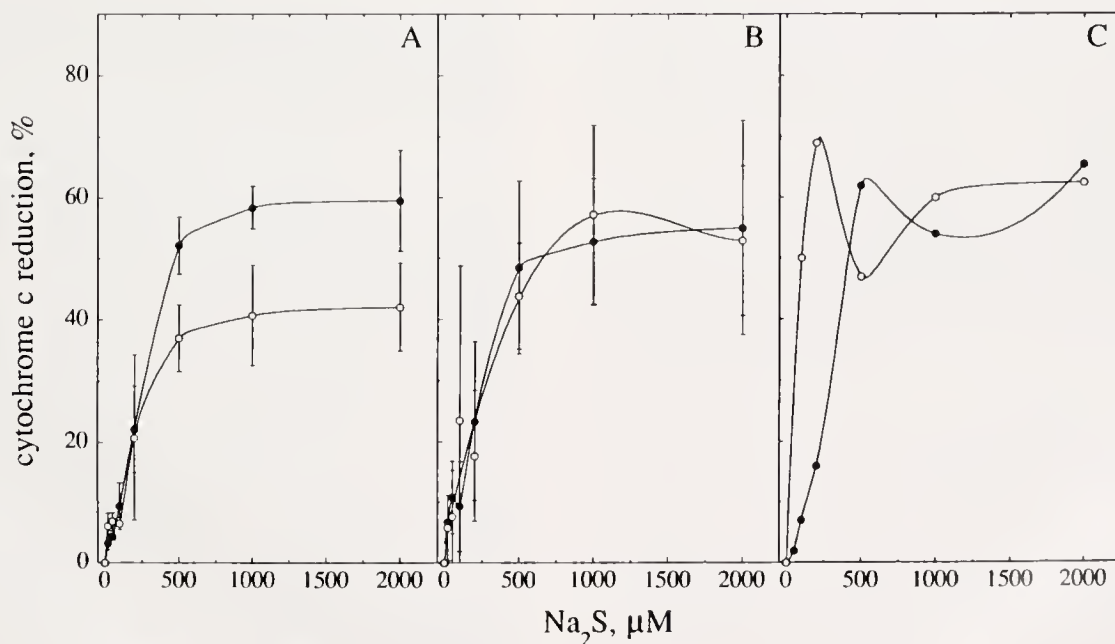


Figure 4. Percentage reduction of cytochrome *c* in mussel gills in the presence (open circles) and absence (closed circles) of serotonin as a function of sulfide concentration. (A) Gills of freshly collected *Geukensia demissa*. (B) Gills of sulfide-free *G. demissa*. (C) Gills of *Mytilus edulis*. Values are given as mean $\pm$ SD ($n = 3$) in A and B, and as the average of two experiments in C.

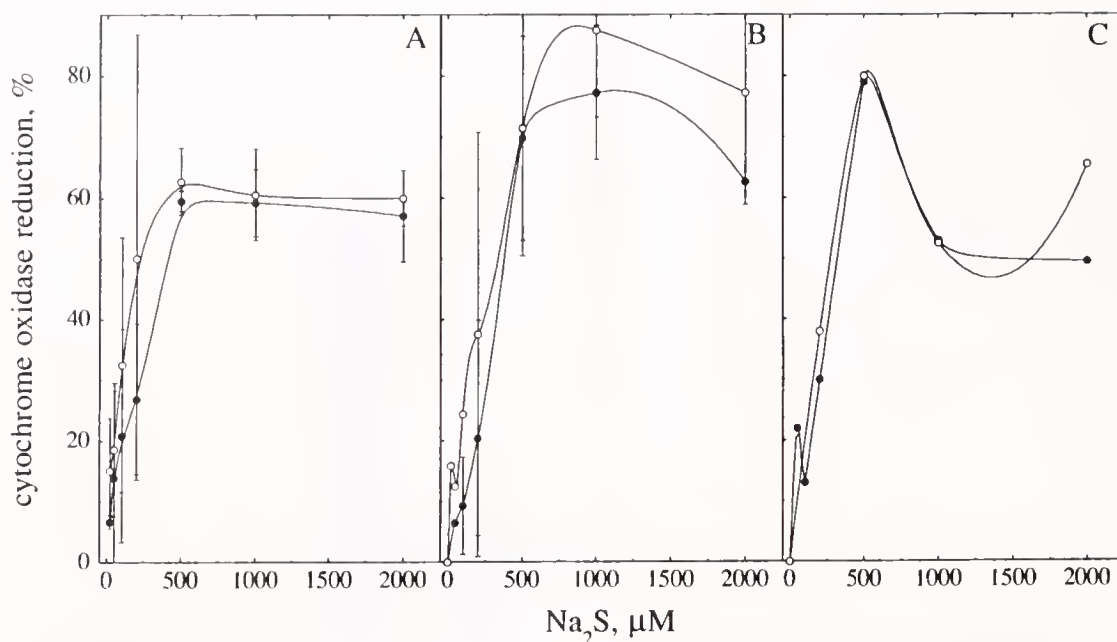


Figure 5. Percentage reduction of cytochrome oxidase (aa_3) in mussel gills in the presence (open circles) and absence (closed circles) of serotonin as a function of sulfide concentration. (A) Gills of freshly collected *Geukensia demissa* (B) Gills of sulfide-free *G. demissa* (C) Gills of *Mytilus edulis*. Values are given as mean $\pm$ SD ($n = 3$) in A and B, and as the average of two experiments in C.

Results from experiments with cyanide and serotonin indicate that part of the sulfide-stimulated oxygen consumption exhibited by *G. demissa* gills may be mitochondrial in nature. Cyanide, an inhibitor of cytochrome oxidase, reduced the rate of sulfide-stimulated oxygen consumption by 61%, indicating that treatment to inhibit mitochondrial electron flux reduces the rate of sulfide oxidation. In buffered distilled water, pH 9 and 25°C, cyanide at 20 mM inhibits spontaneous oxidation of 20 mM sulfide by greater than 90% (Chen and Morris, 1972). Under our experimental conditions of 1 mM cyanide, pH 8, and 20°C, oxidation of 200 μ M sulfide in chambers containing filtered seawater without gill tissue (called background oxygen consumption) was inhibited by only about 40%. Therefore, the effect of cyanide on spontaneous sulfide oxidation in our system is small. Since cyanide inhibited more than 90% of gill oxygen consumption in the absence of sulfide, this respiration appears to occur mostly *via* mitochondria cytochrome oxidase. However, mechanisms responsible for the observed cyanide-insensitive respiration in the presence of sulfide may include a salicylhydroxamic acid (SHAM)-sensitive pathway in which electrons are transferred to oxygen *via* an alternative oxidase, as has been shown to occur in sulfide-exposed mitochondria isolated from the lugworm *Arenicola marina* (Völkel and Greishaber, 1996). Preliminary experiments with *G. demissa* gills show that serotonin-stimulated respiration that is par-

tially inhibited by 1 mM cyanide is then fully inhibited by the addition of 50 μ M SHAM (Kraus and Doeller, unpub.).

The nearly 2-fold increase in oxygen consumption rate exhibited by mussel gills in the presence of serotonin most likely results from the increased energy demand associated with stimulated ciliary beating. If sulfide oxidation is linked to oxidative phosphorylation, with electrons from sulfide being used for ATP production, then the rates of sulfide oxidation should be enhanced when energy demand is higher, as in the presence of serotonin. Our data confirm this prediction. The sulfide-stimulated increase in oxygen consumption rate in gills of freshly collected *G. demissa* was 45% higher in the presence of serotonin than in its absence, indicating that sulfide may participate in ATP production. The proportion of ATP produced from sulfide oxidation is currently being addressed (Gaschen, Kraus, and Doeller, unpub.). Sulfide delivery to gill cells may also be enhanced by the serotonin-stimulated increase in ciliary beating as a result of reduced boundary layers, as discussed by Wright (1979) and Doeller *et al.* (1993) for the enhanced delivery of amino acids and oxygen, respectively, to mussel gills in the presence of serotonin.

In contrast to freshly collected *G. demissa* gills, gills from *G. demissa* maintained without sulfide or gills from *M. galloprovincialis* did not exhibit sulfide stimulation of oxygen consumption rate after treatment with seroto-

nin. In fact, these serotonin-treated gills exhibited inhibition of oxygen consumption at sulfide concentrations that caused slight stimulation of non-serotonin-treated oxygen consumption. Serotonin-mediated enhancement of electron flux through cytochrome oxidase in these gills may make components involved in oxygen-consuming pathways more sensitive to sulfide inhibition; this is analogous to the change in sensitivity to oxygen exhibited by mitochondria under different metabolic states (Wilson *et al.*, 1988).

Changes in the redox state of mitochondrial cytochrome in mussel gills indicate that experimental conditions have caused differential electron flux through the cytochromes. Our data show little change in cytochrome redox state in gills of either *G. demissa* or *M. edulis* at 0–200 μM sulfide, concentrations that greatly stimulate oxygen consumption rate in gills of freshly collected *G. demissa*. Cytochromes were reduced by 50% or higher only at $\geq 500 \mu\text{M}$ sulfide, concentrations at which oxygen consumption rate showed inhibition. At these high sulfide concentrations, serotonin-stimulated gills of freshly collected *G. demissa* exhibit comparatively high rates of oxygen consumption—indicating high electron flux through the electron transport chain—and this may have contributed to the observed redox state of cytochrome *c*. Wilson *et al.* (1988) showed that cytochrome *c* in mitochondria isolated from rat liver exhibits oxygen-dependent redox changes that may influence the rate of oxidative phosphorylation.

Little change in redox state suggests either that the rates of supply and exit of electrons to and from the cytochromes were in balance or that electron flux did not change. If the rates were in balance, then the large increase in the rate of oxygen consumption by *G. demissa* gill may indicate that sulfide oxidation is in part mitochondrial and largely regulated. Oxygen consumption rates in the presence of cyanide and serotonin support the scenario that sulfide oxidation is coupled to ATP production. The possibility also exists that sulfide-stimulated mitochondrial electron flux may be regulated but more or less uncoupled from phosphorylation (O'Brien and Vetter, 1990). On the other hand, if electron flux did not change, then the large increase in the rate of oxygen consumption by *G. demissa* gill must occur *via* pathways not coupled with ATP production.

In summary, the oxygen consumption rate of mussel gills is stimulated by sulfide to an extent that appears to be correlated with environmental exposure to sulfide. Sulfide-stimulated oxygen consumption may be the result of a metabolic pathway, perhaps associated with mitochondria, whose activation may be mediated by environmental sulfide. Whether this process is coupled with ATP production remains unknown, but sulfide-stimulated oxygen consumption by organisms inhabiting

high-sulfide environments most likely represents a mechanism for sulfide detoxification.

Acknowledgments

We thank B. Gaschen, S. Powell, and J. Pino for assistance with collection and maintenance of *Geukensia demissa*, and Brad Siebel for collection of *Mytilus edulis*. *Mytilus galloprovincialis* was supplied by ECOMAR, Santa Barbara, California. This work was supported in part by NSF IBN9219658 grant to JED and DWK.

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Madreporite Function and Fluid Volume Relationships in Sea Urchins

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Abstract. An effort was made to demonstrate an influx of seawater through the madreporites of sea urchins and to evaluate how such an influx, along with osmotic differences and other factors, could contribute to fluid homeostasis. Fluorescent microbeads placed in the medium of *Strongylocentrotus droebachiensis* were taken up into the pore canals and stone canal and distributed (in small numbers) to the distal tube feet, confirming a slow bulk inflow of seawater through the madreporite, where it is partially purified. Probably none of this fluid stream is diverted to the perivisceral coelom (as it is in asteroids), since experiments with *Strongylocentrotus pallidus* showed no significant movement of a soluble fluorescent tracer into that compartment. The osmotic concentrations of the perivisceral coelomic fluids in these two species, and in *Strongylocentrotus franciscanus*, were higher than that of ambient seawater by 2.66 ± 0.39 mosmol kg^{-1} (mean $\pm$ SE). That small hyperosmoticity, along with the net hydrostatic pressure differences induced by the flexing peristome, probably stabilizes body fluid volume. Likewise, fluid in the tube feet of *S. franciscanus* is elevated by 7.94 ± 1.04 mosmol kg^{-1} above seawater, which should contribute to their inflation. Blockage of the madreporite does not lead to an obviously reduced activity of the tube feet, but over the long term, an influx through the madreporite is necessary. Specimens of *S. droebachiensis* with an obstructed madreporite, fed *ad libitum*, had significantly ($P < 0.006$) reduced gut contents vs controls after 28 days, indicating impaired movement or feeding; and the body weights (*i.e.*, volumes) of unfed specimens were significantly ($P < 0.013$) more reduced after 21 days. Com-

pared to starfish, the rigid test of sea urchins reduces the need for an influx of seawater through the madreporite, but some small admission is still essential.

Introduction

Sea urchins, like many other echinoderms, possess a conspicuous sieve-like opening to the exterior, the madreporite. This structure might be expected to have a function in fluid volume regulation, but no such role has ever been demonstrated. The madreporite is attached to a ciliated "stone canal" that connects through a series of passages to the animal's fluid-filled appendages, the tube feet. Together, these structures constitute the water vascular system, an important distinguishing feature of the phylum.

Observers often assume that seawater is drawn in through the madreporite by the stone canal cilia to hydraulically extend the tube feet. If, however, the connection to the madreporite is removed, the tube feet still continue to function for a long time; I have observed tube feet on broken pieces of sea urchin tests to remain distended and active for days. Further, the fluid within the water vascular system (ambulacral fluid, or AF) of echinoderms is not exactly identical in composition to seawater (Robertson, 1949; Binyon, 1964, 1966, 1976; Prusch, 1977; Ferguson, 1987).

Sea urchins have not been studied adequately, but precise osmotic measurements have been made on the fluid compartments of starfish (asteroids). Ambulacral fluid from starfish is sufficiently hyperosmotic to ambient seawater (about 6 mosmol kg^{-1}) that some water would be drawn into this compartment from the medium, as well as from the perivisceral coelomic fluid (PCF) which is variably 1.5 mosmol kg^{-1} more concentrated than sea-

water (Ferguson, 1990a). But in addition to this osmotic uptake, studies with fluorescent microbeads demonstrate that seawater does flow into the madreporite pores of asteroids (Ferguson, 1990b) and, to a lesser extent, of ophiuroids (Ferguson, 1995), and that it is distributed to peripheral parts of the water vascular system. When soluble dextran tracer was used to measure the flux of seawater into a starfish (*Echinaster graninicola*) through the madreporite, the rate was found to be about 5.5% of the body weight per day (Ferguson, 1989). However, this study also revealed that much of this inflow was diverted from the water vascular system to the perivisceral coelom, thus maintaining the fluid volume of the flexible body. (The Tiedemann's bodies that bulge from the ring canal of asteroids into the perivisceral coelomic space were probably the main route of the diversion (Ferguson, 1990b).) Further investigation showed that some starfish, such as the intertidal *Pisaster ochraceus* with its nearly impermeable integument, appear to rely heavily on transmadreporitic entry of seawater to maintain fluid volume (Ferguson, 1992), whereas others, such as the soft-bodied *Pycnopodia helianthoides*, may be proportionally more dependent on osmotic entry of seawater through a thin, permeable body wall (Ferguson 1990a, 1994). A balance between physical uptake of seawater through the madreporite and osmotic uptake through the integument appears to be typical for asteroids.

Sea urchins (regular echinoids), like asteroids, also have prominent madreporites, but their bodies are rigid. This latter feature would seem, at first, to obviate the need for most adjustments of body fluid volume or for any special mechanism (such as the madreporite) to take up seawater. On the other hand, the flexible peristome may allow for more variation in body fluid volume than has been appreciated, creating a potential need for fluid uptake. Or, the body fluid osmotic relationships of sea urchins may not be the same as those described in starfish (Ferguson, 1990a, 1992), and not adequate to maintain fluid homeostasis. So there is much uncertainty, and unfortunately literature bearing on these issues is desultory and often conflicting.

The most concerted previous attempt to evaluate the role of the madreporite in sea urchins was that of Fechter (1965; see review of Nichols, 1966), who glued small capillary tubes to the madreporites of five urchins (*Echinus esculentus*) so that, by observing the movement of tiny air bubbles in the tubes, he could measure any influx or efflux of fluid through them. Though his apparatus was reported as sensitive to a change of $0.8 \mu\text{l}$, he could detect no net movement of fluid over 24 h. Nevertheless, strong mechanical or chemical stimulation would cause all of the tube feet to contract simultaneously for a prolonged period, and a "slow" fluid outflow totaling 4 to $5 \mu\text{l}$ was seen. During such episodes, the PCF hydrostatic pressure

rose 15–18 mm water (about 150–180 Pa) and remained elevated for about 10 h. Through these and other experiments, as well as on theoretical grounds, Fechter concluded that the primary role of the madreporite is to maintain an even pressure balance between the AF and ambient seawater, so that when the tube feet contract en masse, the displacement of their associated ampullae into the perivisceral coelom would not impart too much pressure on the peristome. Although a limited volume of fluid can be released through the madreporite, as Fechter (1965) suggested, prolonged contraction of all the tube feet is not natural; normally (except for momentary responses) when some tube feet contract, others are extending. Fechter's (1965) failure to see any seawater entry into the madreporite is important, but should be verified.

A more detailed study of the internal fluid pressures of *Strongylocentrotus purpuratus* and *Lytechinus variegatus* was carried out by Ellers and Telford (1992). They inserted a hypodermic needle attached to a pressure transducer through the peristome and recorded the pressure fluctuations associated with simultaneous contractions of the tube feet, but these were brief and only about 4% of the magnitude reported by Fechter (1965). Periodic peristomial movements (induced by the lantern) produced a fluctuating pressure change within the perivisceral coelom of about the same magnitude; the internal pressure tended to be negative (to -8.2 Pa) 70% of the time. Although not mentioned by them, this important finding points to another probable mechanism of PCF homeostasis—pressure filtration of water into the perivisceral coelom from higher pressure areas, particularly from the gut and the tube feet ampullae. Their study led Ellers and Telford (1992) to question Fechter's argument that the madreporite is primarily involved in acute pressure equalization.

Fechter's contention is, furthermore, not consistent with the anatomy of the madreporite and its associated stone canal. The form of the madreporite does not suggest a simple "relief valve." In sea urchins the madreporite typically consists of 300–400 pore canals partially filled with cilia that tend to forcefully exclude particles (Tamori *et al.*, 1996). The inside diameter of each pore is about $21 \mu\text{m}$, which is variable because the pore can contract to less than half its resting size in response to acetylcholine (Takahashi and Tamori, 1988; Takahashi *et al.*, 1991; Tamori *et al.*, 1996). Thus, the total cross-sectional space of the madreporite openings is only about 0.12 mm^2 . The normal fluid exchanges through such a restricted opening are likely to be too small to affect the fluid volume of the animal except, perhaps, over the long term. Nevertheless, fairly large fluid volume changes (milliliters) can take place in sea urchins within hours in response to osmotic challenges (Lange, 1964; Stickle and

Ahokas, 1974). Logic suggests that this last adjustment must involve an initial displacement of the peristome, followed by rapid water and ion movements across the gut and other permeable surfaces. Moreover, in preliminary work leading to the present investigation, sea urchins (*Strongylocentrotus pallidus* and *S. purpuratus*) with obstructed madreporites were repeatedly able to return to a near-normal body weight (and volume) within hours after removal of 1–3 ml of their PCF, clearly showing that the madreporite is not needed for acute large-scale volume changes.

Thus, the functions of the prominent madreporite system of sea urchins remain unknown, and the normal osmotic differences that might exist between their various body fluids and the media have not been accurately measured. In this study methods previously applied to asteroids are used to examine two questions. First, does seawater enter the sea urchin madreporite, and if it does, is the quantity sufficient either to affect the function of the tube feet or to stabilize body fluid volume? Second, are osmotic differences between the internal fluids of sea urchins and the outside medium consistent, and could they contribute to inflation of the tube feet or to augmentation of general body fluid?

Materials and Methods

Treatment of specimens

Work was conducted on four congeneric species of sea urchins collected from waters around Friday Harbor, Washington: *Strongylocentrotus purpuratus*, *S. pallidus*, *S. droebachiensis*, and *S. franciscanus*. They were kept in shallow tanks with flowing seawater and fed *ad libitum* with kelp picked up along the shore. In some cases, the madreporites of “test” animals were obstructed by first scraping the structure with a needle and blotting up the soft tissue, and then sealing the area with freshly mixed, finely ground hydraulic cement. After the cement had hardened for about 10 min, the animals were returned to seawater. These plugs were tolerated very well, and they showed no sign of failure over the course of any of the experiments.

Fluorescent microbead experiments

Influxes of seawater through the madreporite and into the water vascular system were demonstrated as follows. Two small specimens of *S. droebachiensis* (3.1 and 3.4 g) were selected and placed for 5 days in a dish of aerated seawater (250 ml) to which was added 1 ml of a suspension of 0.2 μm YG “Fluoresbrite” carboxylate beads (Polysciences, Inc.). Then, after they were rinsed in seawater, the animals were cut into several parts to facilitate further handling. These were fixed overnight in 10% for-

malin and decalcified under refrigeration for 10 days in several changes of 5% EDTA and 10% formalin. After rinsing in tap water, pieces were selected and trimmed to suitable size for sectioning (3–5 mm diameter). Attention was focused on pieces containing the madreporite complex, the aboral half of the stone canal, and representative parts of the aboral and oral body walls with tube feet and ampullae attached. These were frozen onto stubs with Tissue-Tek OTC compound, sectioned in a cryostat to 20–50 μm , picked up on glass slides, mounted with cover slips over glycerine jelly, and examined under epifluorescence using a Nikon system. Only animals with intact madreporites were used for these observations; obstructing the madreporite would have destroyed the major area to be studied and would have precluded sectioning. Further, previous studies on asteroids and ophiuroids (Ferguson, 1990b, 1995) suggested that particles could enter the water vascular passages only with bulk flow of seawater through the madreporite pores.

Soluble fluorescent tracer experiments

An effort was made to quantify the influx of seawater through the madreporite with the soluble, high molecular weight tracer, fluorescein isothiocyanate dextran (FID), as was previously done with asteroids (Ferguson, 1989, 1994). To accomplish this, 100 mg FID (73,100) (Sigma Chemical) was dissolved in dishes of gently bubbled seawater (800 ml) containing pairs (one “test” and one unaltered “control”) of smallish *S. pallidus* (81–155 g). At 24-h intervals, samples of 0.5 ml PCF were taken from the sea urchins through a 26-gauge hypodermic needle inserted through the peristome. Unlike asteroids, an adequate AF sample could not be obtained from the small lamellar ampullae of these animals, and larger sea urchins (including *S. franciscanus*) require so much medium that work with them is impractical. For analysis of the PCF, the collected fluid was centrifuged, and 0.1-ml aliquots of the supernatant were vortexed in tubes with 3.5 ml phosphate buffer. The fluorescence of the samples in these tubes was read in a Turner fluorimeter equipped with 2A and 47B primary filters and a 2A-12 secondary filter. Measurements were compared with samples of medium that had been similarly processed, as well as samples of medium that were diluted 1:10. This method can detect FID in the PCF to concentrations about 0.25% that of the medium.

Long-term effects of madreporite obstruction

Two sets of experiments tested the effect of long-term obstruction of the madreporite on behavior, tube feet activity, and maintenance of body fluid volume. It was assumed that small, gradual adjustments in the volume of individual sea urchins would be observable as systematic

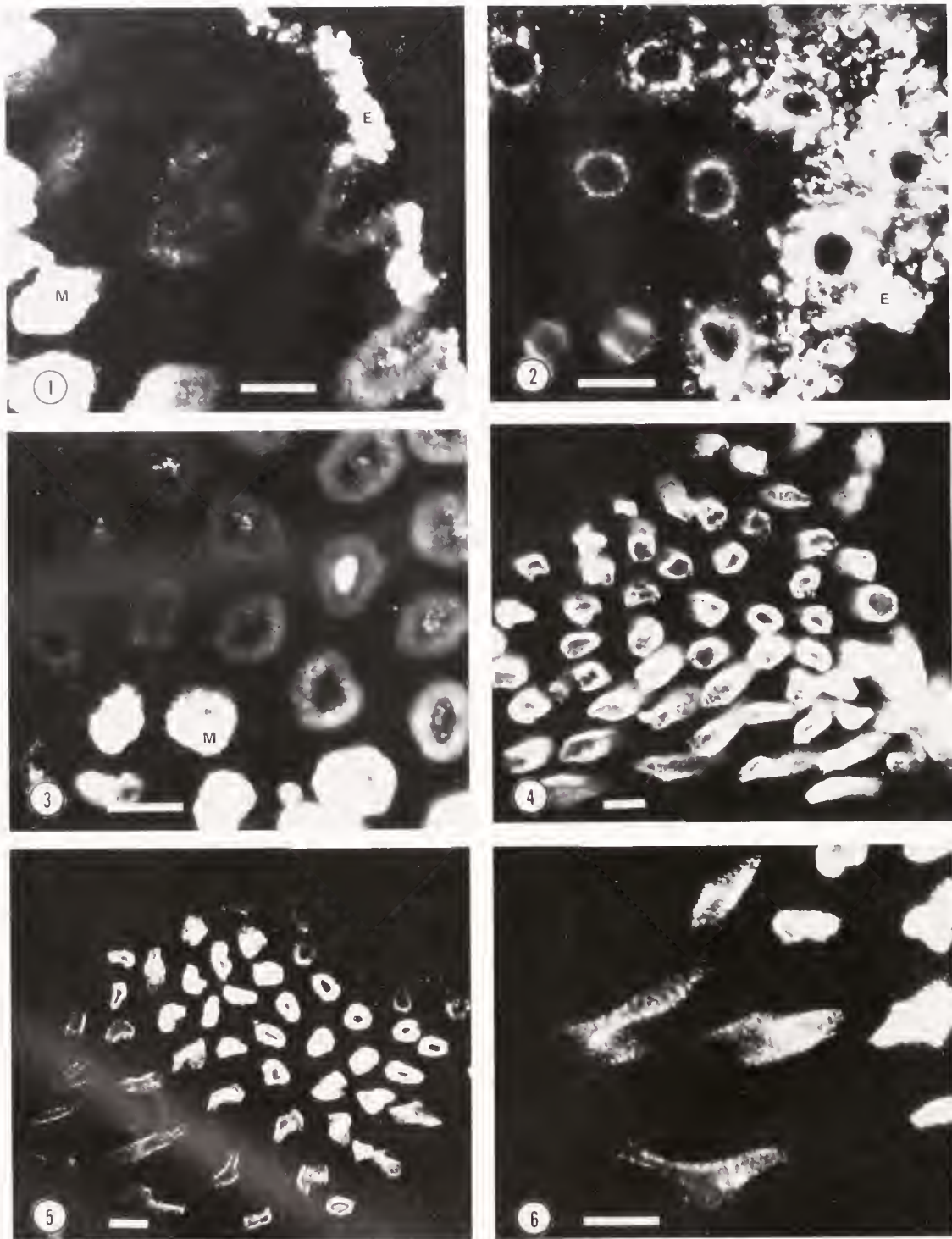


Figure 1. This and the other photographs (Figs. 2–12) show epifluorescent views of unstained sections from two specimens of *Strongylocentrotus droebachiensis* exposed for 5 days to 0.2- μ m fluorescent beads suspended in the medium. Unless otherwise stated, the thickness of sectioning is about 20 μ m. The photograph shows the opening of madreporite pores to the exterior (middle and lower right). The epidermal cells (E) are filled with beads up to the entrance into the pore canals. Some pore canal cells contain beads, and such cells suddenly become much more abundant midway down the canal (M). All scale bars = 50 μ m.

Figure 2. The periphery of the madreporite with the epidermis (E) on right and sections through slightly deeper parts of pores on left. Beads are abundant in pore cells at the entrance of the canals and then become more sparse just inside.

variations in their wet body weights, and be due mainly to the level of inflation of the tube feet, or to adjustments in the position of the peristome. Matched test and control groups of *S. droebachiensis* were selected and observed for several weeks. In one case the animals were fed, and in the other they were not. By using a standardized weighing procedure in which animals were allowed to pre-drain for 2 min on paper towels, daily weight measurements accurate to about 0.1 g were obtained. From a series of such measurements on an individual, a least squares linear regression yielded an average daily change, which was expressed as the percentage of net daily variation from the mean body weight over the period. This deviation was referred to as the "change in body weight index" or Δ WI.

At the conclusion of the experiments, a more specific measurement of the water content of the body parts was made as follows: First, a specimen was inverted for 15 min over a beaker, and any fluid released from its anus was collected. An incision into the body cavity was then made through the equator of the body and the PCF drained out and collected. The animal was then dissected onto tared weighing trays, with the body wall and lantern, the gonads, and the remaining gut separated out. Each collection of tissues was weighed and then dried to constancy (about 24 h) at 75°C, and reweighed. Body water index (BWI) was calculated as the percentage of the wet body weight of the intact animal represented by the drained PCF plus the difference between the total wet and dry weights of parts other than the gut. Gut water index (GWI) similarly was taken as the percentage of the intact animal's wet weight represented by the weight of the collected anal water plus the gut water (difference between wet and dry weights of gut tissue); the water content of any food or feces within the gut was included. The osmotic concentrations of the collected PCF of the animals were also monitored.

Osmotic difference between body fluids and ambient seawater

Finally, osmotic comparisons were made between the body fluids of separate groups of sea urchins and their

ambient seawater. For the PCF, about 0.5 ml of fluid was removed from the body cavity with a 26-gauge syringe needle inserted diagonally through the anus, and was immediately placed in a capped microcentrifuge tube. After flushing the syringe with seawater, an equivalent "reference" sample of ambient seawater was taken and placed in a similar tube. Both samples were then centrifuged (5 min) and 10- μ l aliquots were then analyzed in a vapor pressure osmometer (Wescor 5500). To compensate for any drift in the instrument, the reference seawater sample was analyzed first with three successive replicates on the sample, which remained sealed in the instrument chamber. The PCF was analyzed next in the same way, followed by another set of analyses on the reference seawater. The mean of the six reference seawater measurements was then subtracted from the mean of the three PCF measurements to yield the osmotic difference. Specimens of *S. franciscanus* were sufficiently large that individual tube feet could be seized with a hemostat, cut off, and drained into a centrifuge tube, and their ambulacral fluid (AF) analyzed in the same way. Since these procedures were designed to measure small osmotic differences between body fluids and ambient seawater (a few mosmol kg⁻¹), the precision of the method was determined by analyzing in the same manner 12 replicates of separate seawater samples substituted for the body fluids. These had a standard deviation of ± 2.24 mosmol kg⁻¹.

Statistics

The effects of obstructing the madreporite on Δ WI, BWI, and GWI of test and control animals were evaluated with the Mann-Whitney *U* test, using the *z* statistic to estimate significance. A correction for multiple tests for the three procedures may be made using the Bonferroni procedure, in which the selected confidence limit for a single test is divided by the number of tests. In that case, the 95% confidence limit would be set as $P < 0.017$. In the osmotic studies, the mean differences between body fluids and ambient seawater were evaluated with a Student's *t* test.

Figure 3. Transition to the middle region of the pore canals. In the outer portion, beads are primarily in the lumen (upper right of photograph) or apical part of pore cells (middle and upper left). In the middle region of the pore canal (M) beads are taken up very heavily into all portions of the pore cells.

Figure 4. Middle region of pore canals printed with reduced exposure to show, in detail, the heavy incorporation of beads into the pore canal cells at this level. Section is about 50 μ m thick.

Figure 5. Lower portion of madreporite pore canals. As the canals extend (lower left) towards their confluence to form the sub-madreporite ampulla, their cells contain fewer beads than do those in the mid-region of the canal (center).

Figure 6. Cells in the lower region of the pore canals contain fewer beads than those in the middle region (upper right).

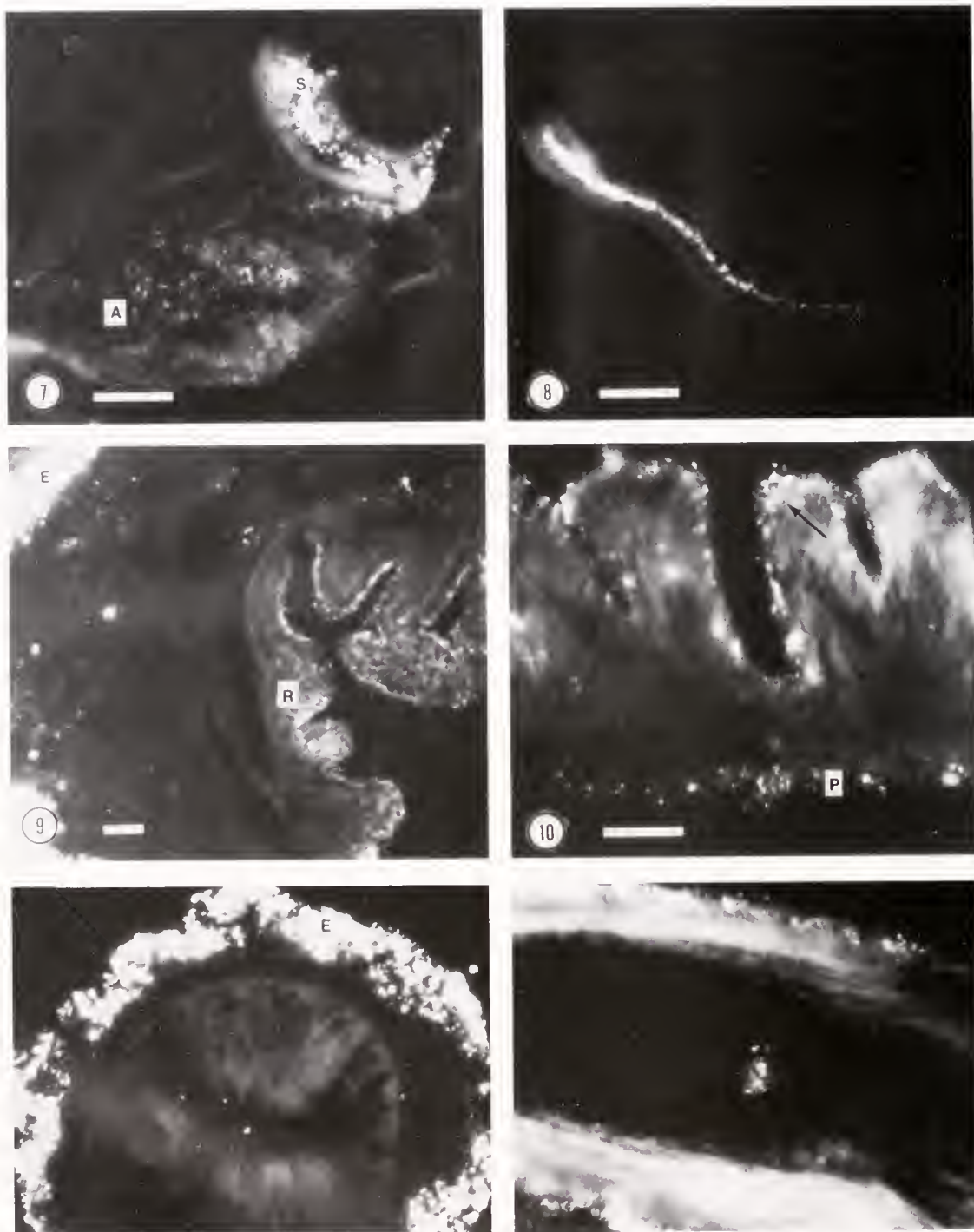


Figure 7. Upper stone canal (s) (near the madreporite) with many beads in its lumen. Some beads may also be seen in the adjacent upper region of the axial organ (A), probably mostly in coelomocytes.

Figure 8. A stringlike conglomerate of beads and perhaps other material within the lumen of the stone canal in a region below that shown in Fig. 7.

Figure 9. Edge of the rectum (R) with a piece of epidermis (E) showing on upper and lower left. Some beads are on the surface of the rectal lining and a few are in its surrounding peritoneum.

Figure 10. Magnified view of the rectum showing beads on its inner surface (arrow) and some in the peritoneum (P). Beads were not found very often elsewhere in the perivisceral cavity, so these may be in the process of being excreted, perhaps by coelomocytes.

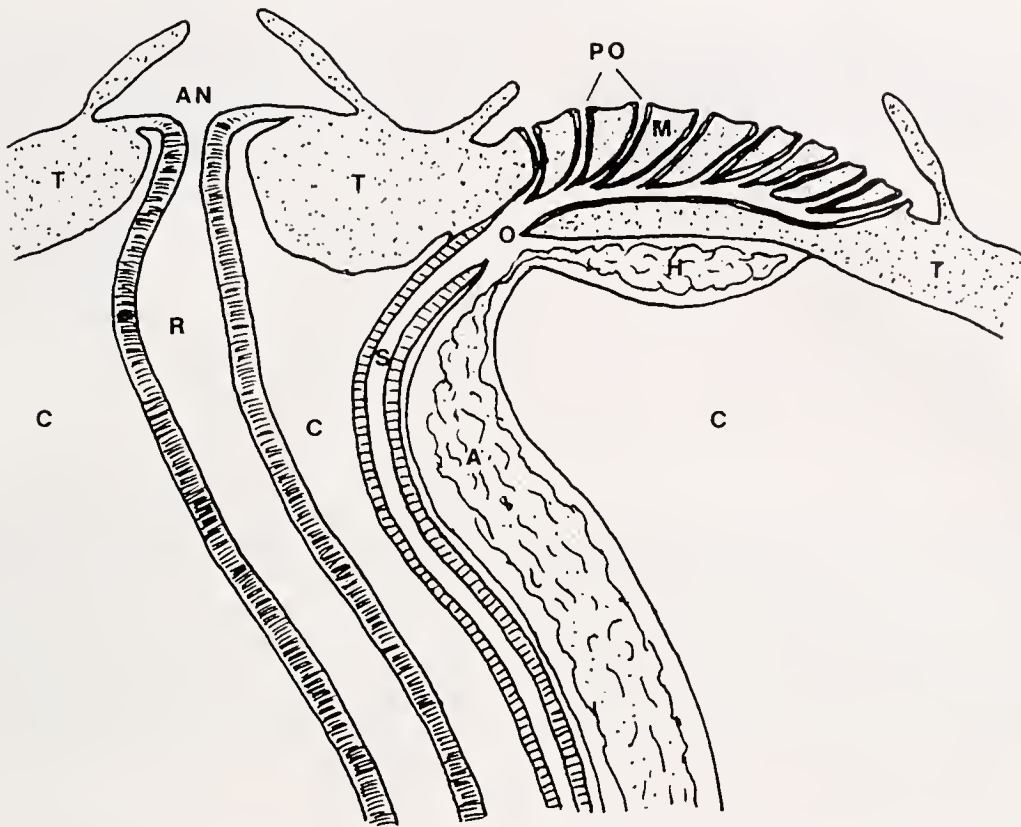


Figure 13. Diagram of the madreporite region. A, axial organ; C, perivisceral coelom; H, head process of axial organ; M, madreporite; O, opening near the upper end of stone canal, between the region of confluence of the pore canals (sub-madreporite ampulla) and the axial sinus; R, rectum; T, testis; AN, anus; PO, madreporite pore canal openings.

Results

Uptake of fluorescent microbeads

As was observed in previous studies on asteroids and ophiuroids (Ferguson, 1990b, 1995), microbeads were taken up extensively into the exposed epidermis of the two urchins examined. Substantial numbers were also found within the water vascular system, especially in the madreporite and stone canal (Figs. 1–12). Figure 13 provides a diagrammatic orientation to the madreporite region of the body. In the madreporite, some beads were seen in the lumen of the outer passages of the fine pore canals, and some within the pore cells, most concentrated in their apical portion (Figs. 1–3). In the mid-region of the canals, numerous beads were found

throughout the pore cells (Figs. 3–5). At the lower end of the pore canals, the abundance of beads retained diminished markedly as the canals extended towards the region of their confluence (*i.e.*, the sub-madreporite ampulla) and the stone canal beyond (Figs. 5, 6). In the stone canal itself, a thick stringlike aggregate of beads and perhaps other material extended down the lumen (Figs. 7, 8). A few beads were also seen in the upper axial organ (Fig. 7), in the lumen of the rectum (found attached to the madreporite pieces that were sectioned), and in the peritoneum (or coelomocytes) near the rectum (Figs. 9, 10). Some beads were found in the lumen of tube feet, especially in the lower (oral) region of the body (Figs. 11, 12), but they were not plentiful. Many, perhaps most, tube foot sections failed to reveal any beads in the lumen.

Figure 11. Cross section of a tube foot from lower (oral) portion of the body. An abundance of beads is evident in the epidermis (E), but several free beads can also be seen in the central lumen.

Figure 12. Longitudinal section through another tube foot in the same region. Beads are seen in a clump of coelomocytes within the lumen.

Table I

Levels of FID (percentage of medium concentration) in the PCF of madreporite obstructed (Test) and unaltered (Control) *Strongylocentrotus pallidus* after incubation in FID seawater

	Weight (g)	Days in medium				
		1	2	3	4	5
Test	87.5	0	0	0	0	0
	112.4	0				
	155.3	0				
Control	80.7	0	0	0	0	0
	94.7	0				
	116.1	0				

Uptake of FID

In three attempts with test and control pairs of *S. pallidus* exposed to high levels of FID in the medium for 24 h (Table I), measurable buildup of the tracer could not be detected in the PCF. In one case, exposure was continued for an additional 4 days, but the substance still could not be observed in the body fluid. I therefore decided that further tests with the FID method were unwarranted.

Long-term effects of madreporite obstruction

A group of madreporite-obstructed specimens of *S. droebachiensis*, and a control group, were observed for 27 days while they were maintained in an aquarium with running seawater and kelp for food. No differences were noticed in the behavior of the two groups. Their tube feet remained active, and animals in both groups were observed to roam the aquarium and climb the sides, feed on the kelp, and rapidly right themselves if upset. No significant differences were measured in the variation of their daily weights (ΔW) or, at the end of the experiment, in their body water content (BW) or its osmotic level (Table II). However, the gut water index (GWI) of

Table II

Effects on indexes of body weight (ΔW), body water (BW), gut water (GW), and PCF osmotic elevation (ΔPCF_{osm}) of groups of *Strongylocentrotus droebachiensis* fed ad libitum for 27 days: madreporite obstructed (Tests) and unaltered (Controls) specimens (11 pairs)

	Tests	Controls
Mean wt. (g $\pm$ SD)	172.6 $\pm$ 24.4	178.0 $\pm$ 31.2
ΔW (% day ⁻¹ $\pm$ SE)	+0.010 $\pm$ 0.011	-0.014 $\pm$ 0.011
BW (% $\pm$ SE)	77.99 $\pm$ 0.75	77.36 $\pm$ 0.56
GW (% $\pm$ SE)	13.21 $\pm$ 0.55*	16.27 $\pm$ 0.86
ΔPCF_{osm} (mosmol kg ⁻¹ $\pm$ SE)	+2.16 $\pm$ 0.84	+2.77 $\pm$ 0.68

* Difference significant ($P < 0.006$).

Table III

Effects on indexes of body weight (ΔW), body water (BW), gut water (GW), and PCF osmotic elevation (ΔPCF_{osm}) of groups of unfed *Strongylocentrotus droebachiensis* over 21 days: madreporite obstructed (Tests) and unaltered (Controls) specimens (6 pairs)

	Tests	Controls
Mean wt. (g $\pm$ SD)	171.5 $\pm$ 27.4	162.0 $\pm$ 40.5
ΔW (% day ⁻¹ $\pm$ SE)	-0.086 $\pm$ 0.011*	-0.021 $\pm$ 0.015
BW (% $\pm$ SE)	77.00 $\pm$ 1.60	79.57 $\pm$ 0.98
GW (% $\pm$ SE)	7.98 $\pm$ 0.53	6.63 $\pm$ 0.40
ΔPCF_{osm} (mosmol kg ⁻¹ $\pm$ SE)	+3.95 $\pm$ 1.23	+2.67 $\pm$ 0.85

* Difference significant ($P < 0.013$).

the "test" animals was found to be significantly ($P < 0.006$) lower than that of the control group (Table II). When a similar experiment was carried out for 21 days on unfed animals, the gut water indexes remained low in both tests and controls, and not significantly different, but the test animals lost weight significantly more rapidly than the controls ($P < 0.013$) (Table III). Otherwise, no differences were noticed in the behavior or well-being of the two groups.

Osmotic differences from ambient seawater

Precise osmotic measurements were made on the PCF of groups of three species (*S. pallidus*, *S. droebachiensis*, and *S. franciscanus*) and compared to ambient seawater (Table IV). All showed mean osmotic levels significantly above that of their ambient seawater ($P < 0.01$), with a combined mean ($\pm$ SE) elevation of 2.66 ± 0.39 mosmol kg⁻¹. Clean samples of ambulacral fluid were obtained from the large tube feet of *S. franciscanus*, and they had a mean ($\pm$ SE) osmotic level (7.94 ± 1.04 mosmol kg⁻¹), significantly above that of the PCF for this species ($P < 0.01$).

Table IV

Osmotic difference between body fluids of sea urchins and their ambient seawater

Species	Fluid	n	mosmol kg ⁻¹ $\pm$ SE
<i>S. pallidus</i>	PCF	12	+2.86 $\pm$ 0.69
<i>S. droebachiensis</i>	PCF	12	+2.68 $\pm$ 0.77
<i>S. franciscanus</i>	PCF	12	+2.44 $\pm$ 0.59
<i>S. franciscanus</i>	AF	12	+7.94 $\pm$ 1.04
Seawater*		12	-0.54 $\pm$ 0.65

* Repetitive samples analyzed just like body fluids (all body fluids are significantly different from seawater ($P < 0.01$)).

Discussion

These experiments with fluorescent microbeads and soluble tracer (FID) show that in contrast to previous work in asteroids (Ferguson 1989, 1990b) entry of seawater into the madreporite of echinoids is very limited. The continued, apparently unimpaired activity of tube feet even after 27 days of madreporite occlusion further indicates that these structures are inflated mainly by some other means. Certainly their elevated osmotic AF ($+7.94 \text{ mosmol kg}^{-1}$ in *S. franciscanus*) must make a contribution, although redistribution of fluids between body compartments may also be involved. Likewise, the failure to detect significant amounts of FID in the PCF shows that there can be but little transmadreporitic inflow of seawater to generate that fluid. If minor entry did occur, it was completely masked by the natural cleansing processes that must exist within the animals and the limits of sensitivity of the method. In a parallel study in which this method was used on the starfish *Echinaster graminicola*, uptake of FID into the PCF was easily measured (in spite of evident fluid cleansing) and found to be about $1.5 \mu\text{l g}^{-1} \text{ h}^{-1}$, out of a total madreporite influx of $2.3 \mu\text{l g}^{-1} \text{ h}^{-1}$ (Ferguson, 1989). The transmadreporite influx of seawater in starfish thus appears to be directed mainly at keeping the flexible body inflated, a process that is largely unnecessary in sea urchins. Moreover, sea urchins do not have Tiedemann's bodies, which may be asteroid specializations for passing fluid to the PCF. With their rigid test obviating much of the need for fluid volume control, sea urchins appear to rely mainly on a higher PCF osmotic level ($+2.66 \text{ mosmol kg}^{-1}$ reported here) than seen in most asteroids (Ferguson, 1990a), and on a net negative hydrostatic pressure reportedly produced within the test by the movement of the lantern and peristome (Ellers and Telford, 1992).

Is there any consistent flow of seawater at all into the sea urchin madreporite? Recent work by Tamori *et al.* (1996) on isolated madreporite pieces indicates that the pores usually maintain an inward flow that is regulated by changes in their diameter. Cilia within the pores tended to eject particles aborally. The present work shows that a small proportion of minute particles (beads) suspended in the medium can still enter the pore canals of intact animals. These particles are extensively trapped, especially by middle pore cells (Figs. 1–6). Many of the particles that succeed in passing through the length of the pore canals become entangled in a string of material within the lumen of the stone canal (Figs. 7, 8), which is lined with ciliated cells filled with granules (Rehkämper and Welsch, 1988). Nevertheless, a few particles pass by these obstacles and are transported to the lumen of distal tube feet, where they are finally phagocytized by coelomocytes (Figs. 11, 12). Particles and phago-

cytic debris also accumulate to some extent in the axial gland, near the rectum, which may be a site of phagocytic egress (Figs. 7, 9, 10). These observations lead to the conclusion that a very slow bulk flow of seawater passes through the madreporite and down the stone canal to the tube feet, and that the pore cells help to remove foreign material from this entering stream.

Although the FID study was not sensitive enough to show such a slow flow, other data support its existence. In working with the starfish *Pisaster ochraceus*, the transmadreporite seawater influx was estimated from the rate of weight loss in animals with an obstructed madreporite (Ferguson, 1992). That value was $2.2 \mu\text{l g}^{-1} \text{ h}^{-1}$ (which was close to the rate determined from the FID studies on *Echinaster* (Ferguson, 1989)). Using a similar approach, madreporite seawater influx in *S. droebachiensis* can be roughly estimated by comparing the mean weight loss differences (ΔW) in test and control animals (Table III). If all the difference between the two groups was due to inhibited madreporite influx, that influx would amount to about $0.3 \mu\text{l g}^{-1} \text{ h}^{-1}$; it is equal to a maximum total inflow of not more than 0.13 ml of seawater a day into the madreporite of a 200-g sea urchin. This rate is at least 70 times lower than the ones calculated for starfish. Although this approach is not very precise, the order of magnitude of the figure obtained seems consistent with differences seen in the microbead observations, negative FID experiments, and other observations, including the negative findings of Fechter (1965).

Does such a low rate of madreporite seawater entry have any functional significance? The long-term weight-change studies (Tables II and III) give some indication that it might, eventually. Although the differences resulting from madreporite obstruction were small and not noticeable in the behavior of the animals, unfed test specimens gradually became unable to maintain body weight, whereas fed test specimens had a slightly higher gut water index. The reasons for these effects are unknown, but possibilities include a gradually increasing deficit of AF that may have limited the number of tube feet that could be used at any one time; or a reduced PCF volume that might have hampered the animal's ability to protrude its lantern apparatus. These kinds of effects could be related. The water vascular system is connected indirectly to the perivisceral coelom through an opening between the upper end of the stone canal and the adjacent axial sinus (Fig. 13) (*cf.* Jangoux and Schaltin, 1977; Bachmann and Goldschmid, 1978). So deficiencies in fluid content in one compartment might affect the functions of the other, given enough time. Thus, this limited fluid uptake appears to "top off" a fluid balance that is largely met by the osmotic differences between the body fluids and the surrounding seawater, and by net negative peridodic hydrostatic pressures in the perivisceral coelom.

The madreporite may have other functions that are unrelated to fluid volume regulation. These include chemosensing, a passage for entry of pheromonal stimulants, and excretory roles. None of these has as yet been convincingly demonstrated.

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Antibacterial Properties of Isolated Amoebocytes From the Sea Anemone *Actinia equina*

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Abstract. The antimicrobial defenses of anthozoans were investigated *in vitro* by extracting amoebocytes from the mesenteric filaments of the beadlet anemone, *Actinia equina*, and testing for their ability to phagocytose and kill the gram-negative bacterium *Psychrobacter immobilis*. Only the hyaline amoebocytes exhibited phagocytosis *in vitro*, with about 40% seen to ingest one or more bacteria over 45 min. Mixed cultures of viable amoebocytes were further found to produce O_2^- ions and other reactive oxygen species (ROS) after stimulation with phorbol myristate acetate or lipopolysaccharide. Co-incubation of viable amoebocytes with *P. immobilis* for 3 h *in vitro* resulted in reduced growth of the bacterium compared to saline-incubated bacteria, but because the growth of *P. immobilis* was also impaired by lysed control amoebocytes, the contribution made to bacterial killing by ROS could not be evaluated. Instead, as confirmed by additional experiments using lysate supernatants of the amoebocytes, it appears that the cells contain soluble bactericidal factors. The nature of these agents is at present unknown, although preliminary tests indicate that killing is not mediated by lysozyme.

Introduction

The Cnidaria is a large, diverse, and ecologically important phylum. It includes about 9400 species, of which 68% are members of the class Anthozoa (Banister and Campbell, 1985). In common with all animals, anthozoans need to protect themselves against the lethal or debilitating consequences of microbial or parasitic inva-

sion. Indeed, a recent report offers evidence that bleaching, one of the most destructive pathological conditions affecting reef corals, may be caused by bacterial infection (Kushmaro *et al.*, 1996). Concern about the health of ecologically important anthozoans, mainly scleractinian corals (Grigg and Dollar, 1990), has stimulated interest in the way these animals overcome or avoid opportunistic or pathogenic infection. However, unlike their coelomate relatives, anthozoans have received little attention with respect to their anti-microbial and anti-parasitic defenses.

The ability of anthozoans to display discriminatory tissue reactions to foreign grafts has been demonstrated by many workers, including Theodor (1970), Burnet (1971), Bigger and Hildemann (1982), Porter and Targett (1988), Jokiel and Bigger (1994), and Rinkevitch *et al.* (1994). It is also widely accepted that these animals possess populations of wandering amoebocytes within the mesoglea (Young, 1974; Patterson and Landolt, 1979; Van der Vyver, 1981; Shick, 1991), and a few studies have noted that these cells tend to migrate to wounds or grafts *in vivo* (Young, 1974; Patterson and Landolt, 1979; Bigger and Olano, 1994). However, despite the rarity of bacterial infection or parasitization in the tissues of these animals (Patterson and Landolt, 1979; Bigger and Hildemann, 1982), detailed *in vitro* analyses of the phagocytic capability of the amoebocytes in anthozoans have not been undertaken, and the cellular basis of anti-bacterial activity remains largely unknown.

The present study was aimed at determining the phagocytic and bactericidal capability of amoebocytes of anthozoans *in vitro*, using the beadlet anemone, *Actinia equina*, as a convenient experimental host. In particular, experiments were designed to measure the rate of uptake of bacteria by the cells, investigate the production of reactive oxygen species (ROS) by the phago-

Received 21 March 1995; accepted 12 August 1996.

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cytes, and ascertain the ability of the amoebocytes to inhibit bacterial growth *in vitro*. In many animals, O_2^- ions and other ROS generated by the cells during the respiratory burst are known to mediate bacterial killing by phagocytes (Fridovich, 1978; Nathan *et al.*, 1979). The process has been extensively studied in mammals, fish, and molluscs (Segal and Abo, 1993; Secombes *et al.*, 1988; Adema *et al.*, 1991; Pipe, 1992). It has also been reported for crustaceans (Bell and Smith, 1993) and ascidians (Bell and Smith, 1994) but not, as yet, for acoelomate animals.

Materials and Methods

Animals

Specimens of the beadlet anemone, *Actinia equina*, were collected from the lower shore of St Andrews Bay, Scotland, and maintained in a flow-through seawater aquarium (15°–20°C) for 1 week before use.

Extraction of amoebocytes

Amoebocytes were extracted from the mesenteric filaments, located at the base of the coelenteron, by making an incision into the center of the pedal disc and excising the filaments into a sterile 15-ml plastic centrifuge tube (Smith and Hutton, 1995). For the phagocytosis experiments, the filaments were mixed with about 3 ml of homologous fluid drained from the coelenteron and vigorously pipetted to dissociate the amoebocytes. Suspension of the cells in coelenteron fluid was found to be necessary to enhance the ability of the amoebocytes to spread on glass surfaces for the phagocytosis assays (see below). To avoid interference from endogenous antioxidant enzymes or other factors that might be present in coelenteron fluid, it was not used for the other experiments. Instead, the filaments were mixed with 3 ml of sterile 4% ocean salts (OS; Sigma, Poole, Dorset, UK), pH 7.0, and similarly pipetted. In each case, the amoebocytes were then allowed to stand for 5 min to sediment undissociated cells, the supernatant was removed, and the cell number was adjusted to a stock concentration of $5.2 \times 10^4 \text{ ml}^{-1}$ with OS. The amoebocytes were either used immediately or stored on ice for no longer than 30 min.

Bacteria

The gram-negative marine bacterium *Psychrobacter immobilis* (formerly *Moraxella* sp.; NCIMB 308) was used as a convenient test agent. It was chosen because it is easy to culture in the laboratory, is of a size readily distinguishable from intracellular inclusions in phagocytosis assays, and—although not pathogenic for anthozoans—is known to be susceptible to the antibacterial

effects of marine invertebrate tissues or body fluids (Smith and Ratcliffe, 1978; Chisholm and Smith, 1992). It was grown to log phase in marine broth (MB; Difco, Detroit, Michigan) for 24 h at 20°C. Volumes of 15 ml were harvested, centrifuged at $400 \times g$, 4°C (10 min), and washed twice in sterile 3.2% NaCl. The bacteria were then resuspended in OS at a stock concentration of $5 \times 10^8 \text{ ml}^{-1}$.

Measurement of phagocytosis *in vitro*

Amoebocyte suspension in homologous coelenteron fluid was pipetted, in 200- μl volumes, onto clean, pyrogen-free, glass coverslips in sterile tissue-culture-grade six-well plates (Sterilin, Stone, Staffs, UK). The coverslips were incubated at 20°C for 15 min to allow the amoebocytes to attach to the glass surface and spread. Each coverslip was then gently washed with two 1-ml volumes of sterile 0.22- μm -filtered OS and moistened with a fresh 50- μl volume of OS. The cells were challenged with 100 μl of washed bacterial suspension or, for the controls, 100 μl of sterile, filtered OS. The latter acted as an “uptake” control to permit direct visual comparison with the experimental cells. All monolayers were then incubated for 15, 30, or 45 min at 20°C. Uptake was not studied over longer incubation periods because preliminary evaluation of amoebocyte viability on the coverslips by eosin exclusion showed a decline from about 80% at 45 min to about 4% after 60 min (data not shown). The reason for the loss of cell viability on the monolayers at 60 min is unclear. The presence of the bacteria did not appear to be responsible because both control (saline-incubated) and experimental (bacteria-treated) preparations showed the same degree of amoebocyte survival (data not shown). Instead, the amoebocytes apparently spread so extensively on the glass surface that they lost structural integrity. After incubation, the monolayers were gently washed with three 1-ml changes of sterile, filtered OS and observed, unfixed, under phase contrast optics of a Leitz Diaplan microscope. Intracellular bacteria were distinguished from extracellular forms according to the criteria given in Smith and Ratcliffe (1978).

Superoxide anion assay and role of the respiratory burst in bacterial killing

The ability of the amoebocytes to produce O_2^- ions was investigated by using the ferricytochrome *c* reduction assay described by Pick and Mizel (1981). First, amoebocyte viability in OS in plastic microplate wells was checked by eosin exclusion at 0, 30, 60, 120, and 180 min at 20°C. These experiments confirmed that cell survival under these culture conditions remained above 85% for 3 h (data not shown). For the superoxide anion

assay, phorbol myristate acetate (PMA) (Sigma) or lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma) were used as elicitors, and the reactions were carried out in 96-well flat-bottomed microtiter plates, one per animal. For each treatment quadruplicate wells were prepared. All of the chemicals were made up in OS and were added to the wells in the order given: 50 μl of cytochrome *c* (from horse heart; Sigma) (final concentration in the well 64 μM); 25 μl of amoebocytes (final concentration $1.3 \times 10^4 \text{ ml}^{-1}$); 25 μl of OS; and, immediately prior to reading, 25 μl of PMA or LPS. To determine the minimal concentration of the elicitors needed for clearly discernible cytochrome *c* reduction by the amoebocytes, the experiment was carried out using 1, 4, or 8 $\mu\text{g ml}^{-1}$ of PMA or LPS, each in the presence of 0, 1, 4, or 8 $\mu\text{g ml}^{-1}$ catalase (all final concentrations). Controls received 25 μl of OS in place of PMA or LPS. To ensure that no interaction occurred between the different compounds, all reagents were mixed independently with the ferricytochrome *c* at 20°C. The plates were read immediately at 550 nm on a microplate spectrophotometer (Dynatech, Billingshurst, West Sussex, England) against blanks composed of 25 μl amoebocytes and 100 μl OS. Additional readings were taken at 5-min intervals for 30 min. The experiment was repeated for a minimum of five animals and the mean absorbance determined for each treatment.

To establish that the reduction of cytochrome *c* by PMA-stimulated amoebocytes from *A. equina* (see Results below) was due to the production of ROS, additional experiments were run in which 25 μl of superoxide dismutase (SOD; an enzyme that converts O_2^- to H_2O_2), mannitol (a hydroxyl radical scavenger), or trifluoperazine (TFP; an inhibitor of NADPH oxidase)—all obtained from Sigma—was included in the reaction mixtures. The respective final concentrations of these chemicals were 1200 units ml^{-1} , 40 mM, and 10 μM . Preliminary experiments confirmed that amoebocyte viability was unaffected by these reagents at these doses. In each case, the PMA concentration in each well was 8 $\mu\text{g ml}^{-1}$ (final), whereas that of LPS was 1 $\mu\text{g ml}^{-1}$. With LPS, 25 μl catalase (final concentration 1 $\mu\text{g ml}^{-1}$) was also included in the reaction mixture; whereas with PMA the catalase was replaced with OS. These concentrations were selected on the basis of the previous experiments to provide good reduction of ferricytochrome *c* *in vitro* (see above). The inhibitors were always added to the wells before the elicitor, and positive controls received 25 μl of OS in place of the inhibitor. For reagent controls, each chemical was incubated independently with non-stimulated amoebocytes and cytochrome *c*. The effect of the various inhibitors was read at 550 nm after 5 min at 20°C, and the change in absorbance from the resting 0 min level was calculated.

Measurement of bactericidal activity by viable amoebocytes *in vitro*

The ability of isolated amoebocytes from *A. equina* to inhibit the growth of *P. immobilis* *in vitro* was examined using a modification of the microplate assay described by Peck (1985) for macrophages. Fifty microliters of amoebocyte suspension in OS (stock concentration $5 \times 10^4 \text{ ml}^{-1}$) was added to wells of 96-well flat-bottomed microtiter plates. Experimental wells were then given 50 μl of prepared bacterial suspension in OS (diluted to a stock of $2 \times 10^5 \text{ ml}^{-1}$) and 25 μl of MB made to manufacturer's specifications (Difco, Detroit, Michigan); amoebocyte controls received 50 μl of sterile, filtered OS and 25 μl of MB. An additional series of wells (the bacteria controls) contained 50 μl of bacterial suspension, 50 μl of OS, and 25 μl of MB. The plates were incubated for 3 h at 20°C and the amoebocytes were then lysed by addition of 100 μl of a stock solution of 0.2% Tween (Sigma) to each well. This step was included to prevent further generation of ROS by the experimental cells. Next, 50 μl of bacterial suspension (stock solution $2 \times 10^5 \text{ ml}^{-1}$) was added to each amoebocyte control well, with 50 μl of sterile, filtered OS added to each of the experimental wells and each of the bacteria controls. Finally, 150 μl of MB was given to every well to support the growth of surviving bacteria. The absorbance of the wells was read at 570 nm on a microplate spectrophotometer (Dynatech) (time T_0). The plate was incubated for a further 18 h at 20°C and read again at 570 nm (time T_{18}).

Bacterial growth was seen as an increase in absorbance over 18 h at 20°C. The effect of the amoebocytes on bacterial growth was calculated as the survival index (SI). This was derived as follows:

$$\text{SI} = \frac{\text{absorbance at 570 nm at time } T_{18}}{\text{absorbance at 570 nm at time } T_0} \times 100$$

With this notation, SI values greater than 100 indicate growth, whereas those below 100 indicate an antibacterial effect (*i.e.*, growth inhibition or bacterial killing).

Antibacterial activity of amoebocyte lysate supernatant (ALS)

To clarify whether the antibacterial properties of *A. equina* amoebocytes are due to soluble factors or to membrane-bound proteins, the effect of lysate supernatants of the amoebocytes (ALS) on the test bacterium, *P. immobilis*, was examined *in vitro*. Each ALS sample was prepared by homogenizing washed amoebocytes in 3 ml of OS in a glass piston tissue grinder (15 min, 4°C) followed by centrifugation at $42,000 \times g$ (20 min, 4°C) to sediment cell debris. The resulting supernatant (=ALS)

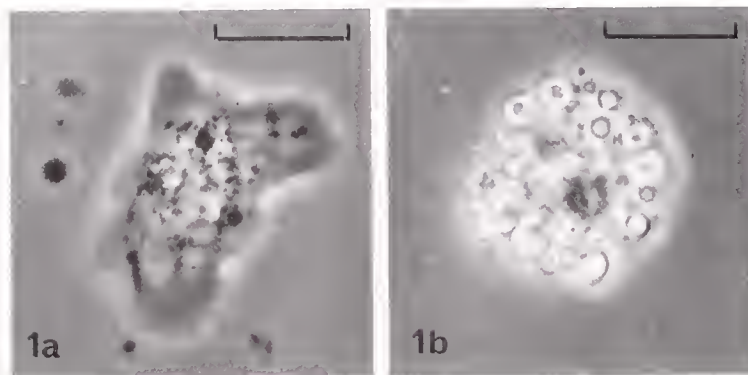


Figure 1. Amoebocytes of *Actinia equina* isolated from the mesenteric filaments after 45-min incubation on glass coverslips. (a) A spread hyaline (phagocytic) type cell containing numerous small, usually nonrefractile, inclusions. Scale bar = 5 μm . (b) An intact granular cell containing many large, highly refractile granules. Scale bar = 5 μm .

was stored on ice until use. Protein in the ALS was determined by the method of Bradford (1976) with bovine serum albumin as standard and routinely adjusted by dilution with OS to 2 mg ml⁻¹ before use. Aliquots (80 μl) of the ALS were then dispensed in quadruplicate wells of a 96-well (flat-bottomed) microtiter plate and mixed with 10 μl of MB and 10 μl of *P. immobilis* (stock concentration 2×10^5 ml⁻¹). For controls, 80 μl of OS was substituted for the ALS. The absorbance of each of the control and experimental wells was read at 570 nm after incubation at 20°C for 0, 3, 6, 12, and 24 h.

Because the bactericidal assays with whole amoebocytes indicated that the response involves lysis (see below), we undertook to ascertain whether the antibacterial properties of *A. equina* ALS are due at least in part to lysozyme activity. A modification of the procedure described in Findlay and Smith (1995) was used. Briefly, ALS samples were prepared as above but in salt-supplemented phosphate-buffered saline (ssPBS; 20 mM Na₂HPO₄·2H₂O; 45 mM KH₂PO₄; 0.1 M NaCl; pH 6.5). They were serially diluted to protein concentrations of 1500, 1000, 500, or 200 μg ml⁻¹, and 100 μl of each dilution was mixed with 900 μl of *Micrococcus luteus* cell walls (Sigma) previously suspended in ssPBS to give an absorbance of 0.5 at 570 nm. An extra tube containing PBS in place of ALS was set up as the negative control. To provide both positive controls and a standard curve by which any lysozyme-like activity in *A. equina* ALS could be quantified, a series of hen egg white lysozyme (Sigma) solutions in ssPBS at the same protein concentrations as the ALS were set up in parallel. All tubes were incubated at 25°C for 30 min with shaking and then read at 600 nm. Lysis in each experimental tube was calculated as the percentage of its positive control. Again the experiment was repeated three times with fresh ALS samples.

Statistical analysis

Differences between each experimental treatment and its control were analyzed by paired Student's *t* test. Differences were considered significant when $P \leq 0.01$ (Sokal and Rohlf, 1981).

Results

Phagocytosis in vitro

Observation of the amoebocyte suspension prepared from the mesenteric filaments of *A. equina* under phase contrast optics revealed the presence of two morphologically distinct cell types. One, the hyaline cells, was seen to contain numerous small, nonrefractile granules (Fig. 1a); the other, the granular cells, contained many large, highly refractile granules (Fig. 1b). The proportion of hyaline to granular cells on the monolayers tended to differ from animal to animal but was usually about 5 : 1 (data not shown). Both types were seen to attach to glass coverslips, but only the hyaline cells exhibited marked spreading, and this was largely dependent upon the presence of coelenteron fluid. The granular cells showed a limited ability to spread on glass, even in the presence of coelenteron fluid. The hyaline cells were also the only amoebocyte type seen to ingest bacteria *in vitro*. After 15 min the percentage of these amoebocytes containing one or more bacteria was $20\% \pm 1.8\%$ (Fig. 2). By 45 min this had increased significantly to a maximum of $39.6\% \pm 1.4\%$ ($P = 0.002$). Intracellular bacteria were not seen in any of the cells on the control, OS-incubated, monolayers.

Superoxide anion assay

Figure 3 shows the effect of different concentrations of PMA or LPS on the *in vitro* reduction of ferricytochrome

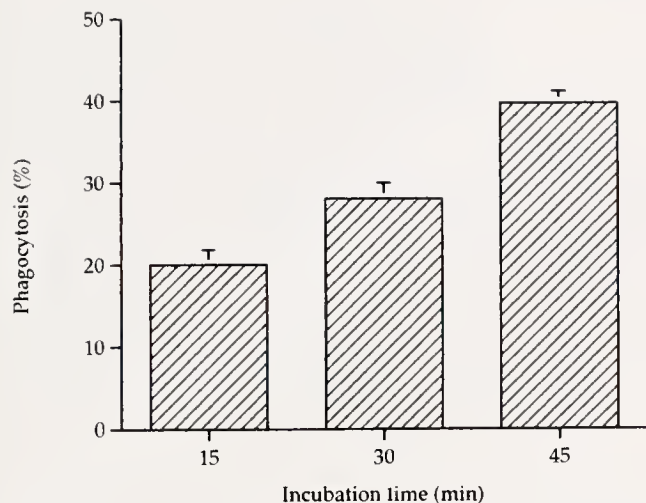


Figure 2. Percentage phagocytosis of *Psychrobacter immobilis* by isolated amoebocytes from *Actinia equina* *in vitro*. Cell monolayers, prepared on glass coverslips, were challenged with 100 μ l of washed *P. immobilis* (stock concentration 5×10^7 in ocean salts [OS]). Controls received 100 μ l of sterile, filtered OS and all cultures were incubated for 15, 30, or 45 min at 20°C. The percentage of cells containing one or more intracellular bacteria was determined by observation of washed, unfixed monolayers under phase contrast optics. Values given are means $\pm$ SE; $n = 5$.

c by the amoebocytes of *A. equina* in the presence of varying catalase concentrations after 5 min. Of all the elicitor-catalase combinations tested, absorbances were highest with PMA at 8 μ g ml⁻¹ without catalase (Fig. 3a), or LPS at 1 μ g ml⁻¹ with catalase at a final concentration of 1 μ g ml⁻¹ (Fig. 3b). This promotional effect of catalase was seen only at a concentration 1 μ g ml⁻¹ and was most marked with 1 μ g ml⁻¹ LPS (Fig. 3b). The reason that catalase at 4 or 8 μ g ml⁻¹ produced an LPS-induced ferricytochrome *c* reduction similar to, or slightly less than, that of the catalase-free condition is unknown. Catalase had only a marginal effect on PMA-elicited cytochrome *c* reduction (Fig. 3a) and therefore was omitted from subsequent experiments with PMA. Neither PMA nor LPS exerted a change in absorbance in control wells lacking *A. equina* amoebocytes (data not shown).

Figure 4 shows the change in absorbance at 550 nm over 30 min for amoebocytes incubated with PMA (8 μ g ml⁻¹ final concentration) or LPS (1 μ g ml⁻¹ final concentration) with or without 1 μ g ml⁻¹ catalase, compared to the controls, in which PMA or LPS and catalase were replaced with OS. The absorbance of the wells at 5 min after treatment with PMA (with or without catalase) or LPS (1 μ g ml⁻¹ plus catalase) was significantly higher than that of their respective OS-incubated controls ($P < 0.0001$ for PMA without catalase, $P < 0.0001$ for PMA with 1 μ g ml⁻¹ catalase, and $P < 0.0001$ for LPS with 1 μ g ml⁻¹ catalase; all compared to the OS-treated samples at 5 min) (Fig. 4a, b). The slight increase in absor-

bance obtained at 5 min with LPS (1 μ g ml⁻¹ without catalase) was not significant at the 5% level compared to the OS controls ($P = 0.643$) (Fig. 4b). Over the next 25 min, the absorbance of all wells declined, and with PMA or LPS, both minus catalase, the absorbances fell to values close to those of the OS-treated controls (Fig. 4a, b). In contrast, PMA or LPS, both plus catalase, produced a much less dramatic decline in absorbance, and the 30-min absorbance values remained significantly above the OS controls ($P < 0.0001$ for PMA; $P < 0.0001$ for LPS) yet below their respective values at 5 min ($P < 0.0001$ for PMA; $P < 0.0001$ for LPS) (Fig. 4a, b).

Thus, exposure of *A. equina* amoebocytes to PMA or LPS seems to result in the reduction of ferricytochrome *c*, indicating that the cells were stimulated to produce ROS through a respiratory burst. The reason for the decline in the absorbance in the control and PMA or LPS (both minus catalase) wells is unknown. The phenomenon may have been due to re-oxidation of ferricytochrome *c*, perhaps by hydrogen peroxide produced by the cells. Certainly, less re-oxidation of cytochrome *c* occurred where catalase, an enzyme that converts hydrogen peroxide to water and molecular oxygen (Vandewalle and Petersen, 1987), was present. In the case of the controls, in which catalase and elicitor were absent, the change in absorbance must have been due to spontaneous oxidation of the substrate over the 30-min incubation period.

In the next series of experiments, inclusion of exogenous SOD in the reaction mixtures was found to significantly reduce the stimulatory effect of PMA on ferricytochrome *c* reduction ($P < 0.038$ for the PMA + SOD-treated cells compared to the cells treated with PMA + OS) (Fig. 5). Because SOD brings about the conversion of O₂⁻ ions to H₂O₂, this observation offers evidence that the reduction in ferricytochrome *c* by *A. equina* amoebocytes induced by PMA is due to the generation of superoxide ions *in vitro*. Likewise, mannitol and TFP significantly abrogated the effect of PMA on the absorbance of the experimental wells ($P < 0.0001$ for each reagent compared to the PMA-treated amoebocytes incubated with OS) (Fig. 5). The effect of these chemicals on PMA-induced reduction of ferricytochrome *c* by the amoebocytes further indicates that the phenomenon involves NADPH oxidase, OH⁻ ions, and H₂O₂.

Bactericidal activity by viable amoebocytes in vitro

Washed *P. immobilis* incubated with viable amoebocytes showed significantly poorer growth than bacteria incubated with OS and MB only ($P < 0.0001$) (Fig. 6). However, because a reduced SI was also derived for the control (Tween 20 lysed) amoebocytes (Fig. 6), it is impossible to conclude that bacterial killing was due to metabolic products generated by viable amoebocytes. In-

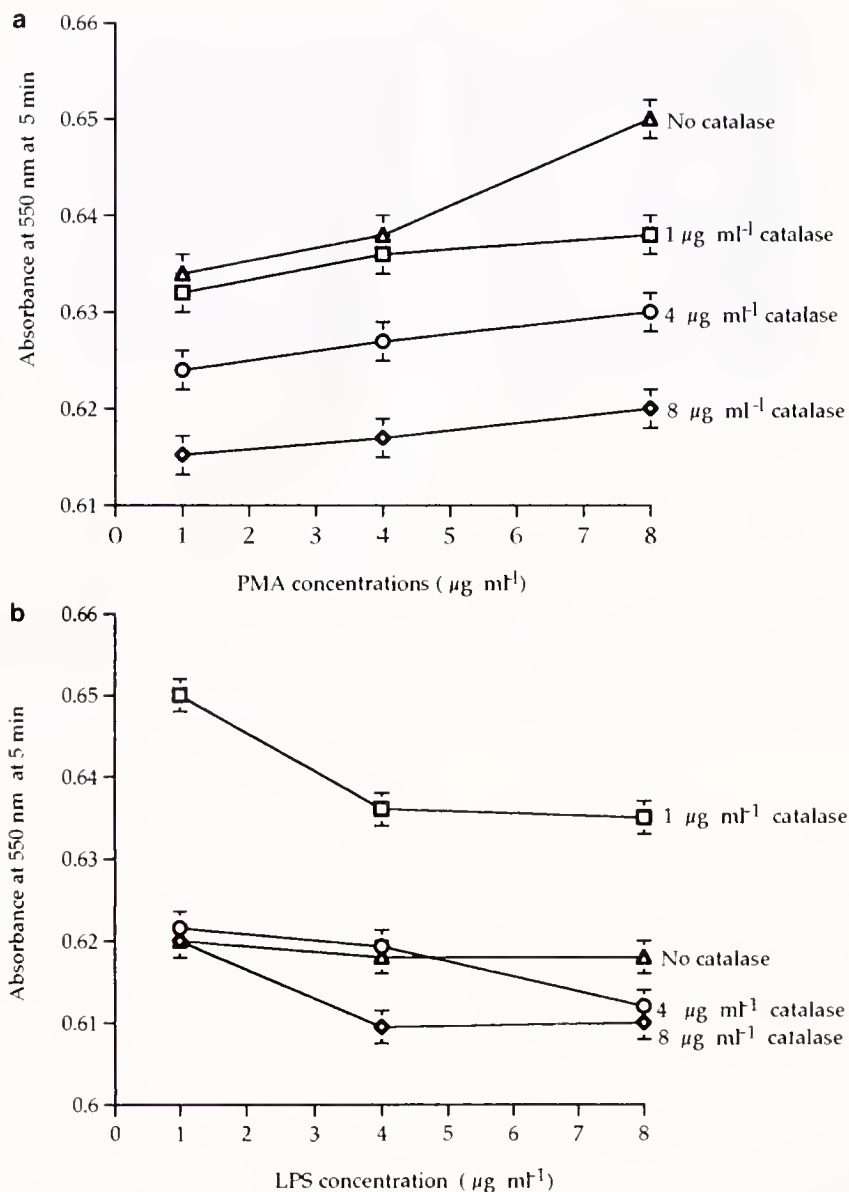


Figure 3. Effect of different phorbol myristate acetate (PMA), lipopolysaccharide (LPS), and catalase concentrations on the reduction of ferricytochrome *c* by isolated amoebocytes from *Actinia equina* *in vitro*. Twenty-five microliters of washed amoebocytes in ocean salts (OS; final concentration $1.3 \times 10^4 \text{ ml}^{-1}$) was incubated with 25 μl of PMA (a) or LPS (b) (both at final concentrations of 1, 4, or 8 $\mu\text{g ml}^{-1}$), 25 μl of catalase (final concentrations 0, 1, 4, or 8 $\mu\text{g ml}^{-1}$), 25 μl of OS and 50 μl of ferricytochrome *c* (final concentration 64 μM). For controls, the PMA or LPS and catalase was replaced by 50 μl of OS and the absorbance of each well was measured at 550 nm after 5 min. Values given are means $\pm$ SE; $n = 5$.

stead, the cells appear to release one or more bactericidal factors upon exposure to Tween 20. Given that the SI for both experimental and control amoebocytes was below 100 ($P < 0.0001$) (Fig. 6), at least one of these factors is a lysin.

Antibacterial activity of ALS

That the antibacterial activity by *A. equina* amoebocytes was due to one or more soluble, rather than mem-

brane-bound, factors was confirmed by the results of the experiments with ALS. Experimental (ALS-incubated) bacteria showed significantly poorer growth than control *P. immobilis* incubated in OS supplemented with MB ($P < 0.0001$ after 18-h incubation at 20°C) (Fig. 7). Unfortunately, this assay failed to confirm that the antibacterial effect of *A. equina* ALS involves lysis, possibly because the rapid growth of surviving bacteria would have masked any reduction in bacterial turbidity

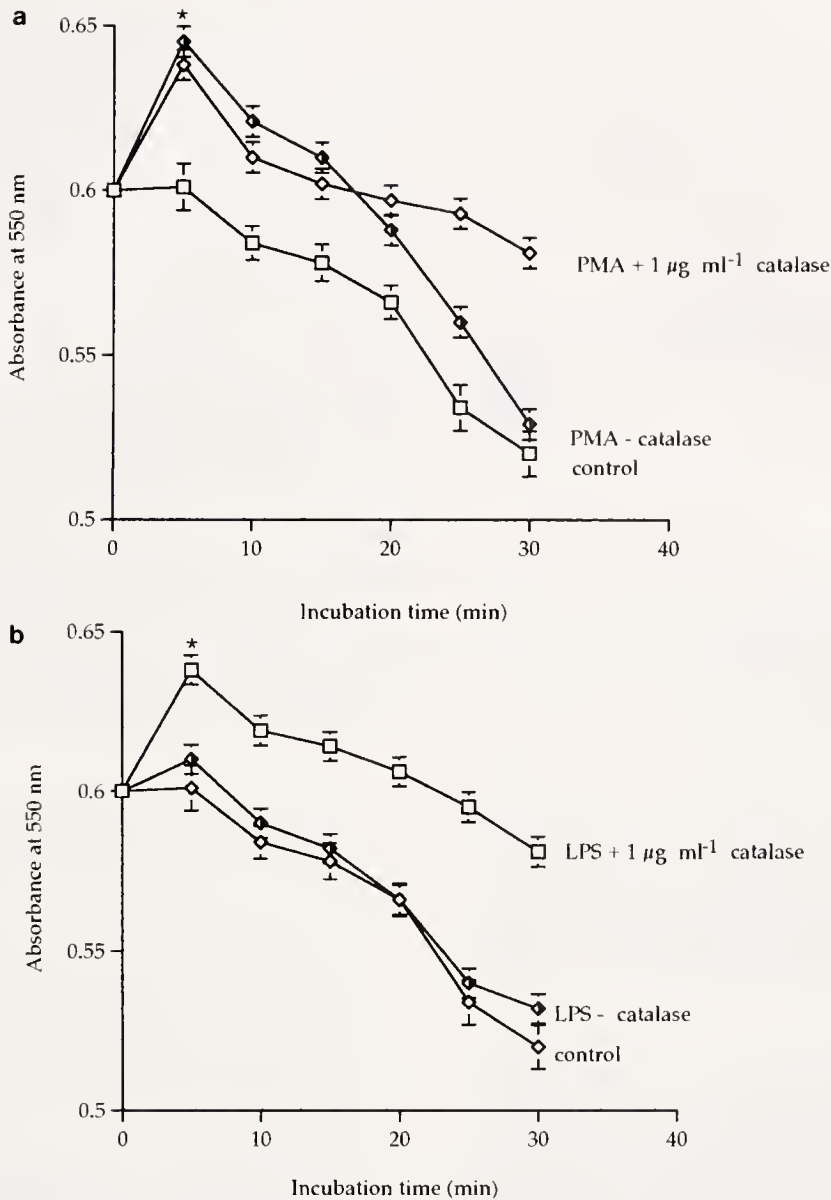


Figure 4. Reduction of ferricytochrome *c* by amoebocytes from *Actinia equina* *in vitro*. Twenty-five microliters of washed cells in ocean salts (OS; final concentration 1.3×10^4 ml⁻¹) was incubated with 25 µl of phorbol myristate acetate (PMA; final concentration $8 \mu\text{g ml}^{-1}$) (a) or lipopolysaccharide (LPS; final concentration $1 \mu\text{g ml}^{-1}$) (b), 25 µl of OS and 50 µl of ferricytochrome *c* (final concentration $64 \mu\text{M}$). For controls, PMA or LPS was replaced with 25 µl of OS. An extra series of wells, in which 25 µl of catalase (final concentration $1 \mu\text{g ml}^{-1}$) was substituted for the OS, was also included for both PMA- and LPS-treated cells to enable the effect of catalase on ferricytochrome *c* reduction to be monitored over 30 min. The absorbance of each well was measured at 550 nm at 5-min intervals for 30 min. Values given are means $\pm$ SE; $n = 5$. An asterisk (*) indicates that differences between experimental and control values are significant at the 1% level.

compared to the time zero controls. Our subsequent experiments to test for lysozyme-like activity also failed to reveal significant lysis of *Micrococcus luteus* cell walls at any of the ALS concentrations tested, indicating that lysozyme is not one of the main agents involved in bacterial killing.

Discussion

The results of this study show that amoebocytes isolated from the mesenteric filaments of *A. equina* are actively phagocytic *in vitro* and produce substances that are able to inhibit the growth of gram negative bacteria

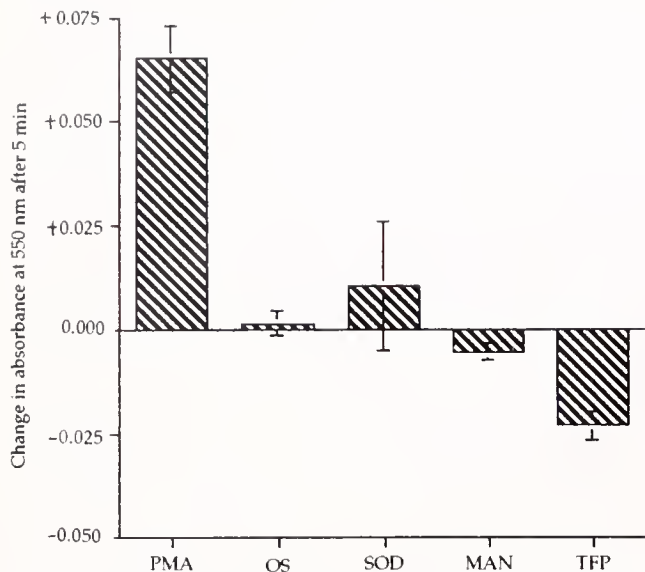


Figure 5. Change in ferricytochrome *c* reduction by phorbol myristate acetate (PMA) stimulated amoebocytes from *Actinia equina* by treatment with superoxide dismutase (SOD), mannitol (MAN), or trifluoperazine (TFP) *in vitro*. Twenty-five microliters of amoebocyte suspension in ocean salts (OS; final concentration $1.3 \times 10^4 \text{ ml}^{-1}$) was challenged with 25 μl of PMA (final concentration $8 \mu\text{g ml}^{-1}$), 50 μl of ferricytochrome *c* (final concentration $64 \mu\text{M}$), and 25 μl of OS (controls) or 25 μl of the appropriate reagent (experimental), at final concentrations of 1200 units ml^{-1} , 40 mM, or 10 μM , respectively. All samples were incubated for 5 min and read at 550 nm. The absorbance of each well was measured at 550 nm after 5 min at 20°C and the change in absorbance from the resting value at 0 min calculated. Values given are means $\pm$ SE; $n = 5$. Differences between the experimental (i.e., SOD-, MAN-, or TFP-treated) and control (i.e., PMA-stimulated) amoebocytes was significant at the 1% level.

within 3 h. There have been few previous analyses of the bactericidal properties of anthozoan tissues and none aimed at examining the mechanism for antibacterial effect. An early study by Burkholder and Burkholder (1958) screened for antimicrobial activity in gorgonians and scleractinian corals; although activity was detected in several species, the authors were unable to identify the tissue responsible or to establish whether activity was mediated by the symbiotic zooxanthellae or the host tissue. Astley and Ratcliffe (1989) examined the antimicrobial and agglutinating properties of mucus from *Metridium senile* but did not consider activity in the tissues. In contrast, a more recent investigation by Kim (1994) was directed towards the antibacterial responses of gorgonian tissues, and although activity was found against non-marine bacteria, the assays were made on whole-body extracts only. To the best of our knowledge, this is the first detailed report confirming the antibacterial capability of anthozoan amoebocytes *in vitro*, and the first to show that large numbers of phagocytically competent amoebocytes can be isolated from the mesenteric filaments.

The absence of a coelom and distinct blood vascular system in anthozoans has tended to exclude these animals from the realms of comparative hematology. In place of circulating blood cells, the wandering amoebocytes of the mesoglea have been regarded as primordial immunocytes responsible for non-self surveillance and host defense (Phillips, 1963; Bigger and Hildemann, 1982), an idea based mainly on observations that these cells accumulate at wound sites or foreign tissue grafts (Young, 1974; Patterson and Landolt, 1979; Bigger and Hildemann, 1982; Bigger and Olano, 1994). Research into the true biological roles and phylogenetic status of the mesogleal amoebocytes is hampered by the small, and often variable, number of the cells in anthozoans and the difficulty of their isolation for *in vitro* analysis (Patterson and Landolt, 1974; Van der Vyver, 1981). The present study avoided these problems by using amoebocyte populations extracted from the mesenteric filaments. The exact relationship of the mesenteric filament amoebocytes to those in the mesoglea is unclear. Authors disagree about the origin and primary role of the amoebocytes in the mesoglea (Chapman, 1974; Young,

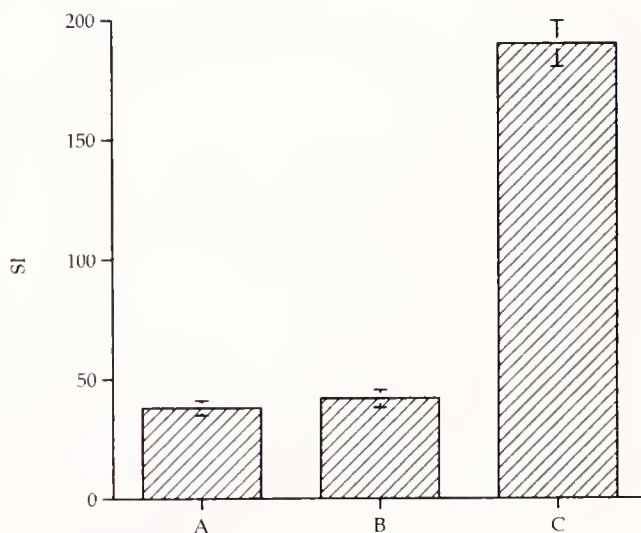


Figure 6. Antibacterial activity of intact, viable amoebocytes from the mesenteric filaments of *Actinia equina* *in vitro*. The experimental wells (A) and the control wells (B) each contained 50 μl of amoebocytes and 25 μl of marine broth (MB). Fifty microliters of *Psychrobacter immobilis* was added to A, whereas 50 μl of ocean salts (OS) was added to B. The bacteria controls (C) comprised 50 μl of *P. immobilis*, 50 μl of OS, and 25 μl of MB. After 3 h each well received 100 μl of Tween 20 (100 μl); 50 μl of OS was then added to A and C, while 50 μl of *P. immobilis* were added to B. Finally, 150 μl of MB was pipetted into every well to support the growth of surviving bacteria, and the samples were incubated for a further 18 h before measurement of turbidity at 570 nm. The survival index (SI) was calculated as described in the Materials and Methods. Values given are means $\pm$ SE; $n = 5$. Differences between A and C are significant at the 1% level, whereas differences between A and B are not significant at the 5% level.

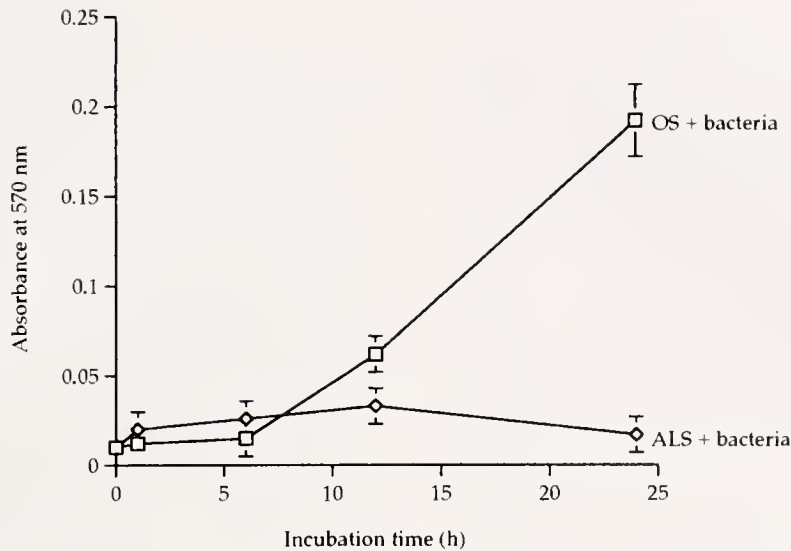


Figure 7. Antibacterial activity of amoebocyte lysate supernatant (ALS) from *Actinia equina* against *Psychrobacter immobilis* *in vivo*. Ten microliters of *P. immobilis* (stock concentration $2 \times 10^5 \text{ ml}^{-1}$) was incubated with 80 μl of ALS (stock protein concentration 2 mg ml^{-1}) and 10 μl of marine broth (MB) at 20°C . For controls, 10 μl of the bacteria was incubated with 80 μl of OS and 10 μl of MB. After 12 h and 24 h, the absorbances were read at 570 nm. Values given are means $\pm$ SE, $n = 5$. Differences between the control and experimental values are significant at the 1% level.

1974; Willmer, 1991), although these cells are thought to participate in nutrient storage, transport, and digestion (Chapman and Pardy, 1972; Van-Praët, 1985; Shick, 1991). Crucially, Young (1974) has proposed that they are important in wound repair and migrate to sites of injury from a reservoir in the endodermal mesenteries. Our light microscopical observations of the mesenteric filament cells of *A. equina* agree with descriptions of amoebocytes in the mesoglea of other anthozoan species (Westfall, 1966; Singer, 1971; Chapman, 1974; Bigger and Hildemann, 1982). We show, unequivocally, that the hyaline amoebocytes, at least, are capable of phagocytosis, thus confirming their potential to sequester and inactivate foreign material from the endoderm. The finding that the mesenteric filament amoebocytes in *A. equina* also exhibit antibacterial activity signifies that they must play a role in host defense, and thus constitute a valuable source of immunocompetent effector cells for *in vitro* analyses.

Interestingly, the presence of two morphological categories of amoebocytes (hyaline and granular cells) noted for *A. equina* in the present investigation is comparable to the situation in most other invertebrate phyla, in which phagocytes and nongranular-type cells occur almost universally (Ratcliffe and Rowley, 1979). In addition, the amoebocytes of *A. equina* appear able to attach to glass surfaces, to exhibit (a variable degree of) cell spreading, and to ingest foreign particles *in vitro*; these features are also shown by certain blood cell types in

many invertebrate species. However, unlike the cells in other groups, in *A. equina* amoebocyte spreading seems to be influenced by the coelenteron fluid. As yet, we do not know how this fluid affects spreading behavior, but unpublished observations in our laboratory indicate that it involves one or more heat-labile, dialyzable, and calcium-dependent "factors." Surprisingly, spreading of the amoebocytes, especially the hyaline cells, on glass surfaces did not appear to be self-limiting as it is with the blood cells of other invertebrates: a sizable proportion of these cells were seen to disintegrate after 45–60 min on the monolayers. To avoid this problem in the superoxide anion and bactericidal assays, where more prolonged incubation times were required, cell spreading—and hence cell disintegration—was minimized by incubating the amoebocytes in OS and in wells of tissue-culture-grade plastic microtiter plates instead of on glass coverslips.

Notwithstanding, the phagocytic vigor of *A. equina* amoebocytes *in vitro* is within the range of values obtained for the phagocytes from coelomate invertebrates cultured under similar conditions. For example, the percentage phagocytosis of *P. immobilis* by amoebocytes from the solitary ascidian *Ciona intestinalis* is about 40% (Smith and Peddie, 1992), and that of phagocytes from the starfish *Asterias rubens* is about 42% (Smith and Saunders, unpub. obs.). The hemocytes from decapod crustaceans tend to show lower rates, at about 15%–20% (Smith and Ratcliffe, 1978; Smith and Söderhäll, 1983), and those of annelids display rates as low as 3%–5% (Fitz-

gerald and Ratcliffe, 1982). Another feature that the amoebocytes of *A. equina* appear to share with the blood cells of other animals is the ability to reduce ferricytochrome *c* following stimulation with PMA or LPS. Moreover, as the response to PMA was found to be impaired by inclusion of SOD, mannitol, or TFP, the reduction of ferricytochrome *c* must be due to the production of superoxide anions and other ROS through an NADPH oxidase-mediated respiratory burst. As far as we are aware, the respiratory burst has not been described previously for a member of the Cnidaria. Importantly, the speed of the response in *A. equina* is comparable with that in other animals; ROS production has been reported to occur within 10 min in oyster phagocytes (Takahashi *et al.*, 1993), 15 min in fish macrophages (Secombes *et al.*, 1988), and 2 min in human neutrophils (Ryan *et al.*, 1990).

For many animals, ROS generated through the respiratory burst have been shown to effect intra- or extracellular microbial killing by the phagocytes (Nathan *et al.*, 1979; Sharp and Secombes, 1993; Segal and Abo, 1993). Whether this is true for *A. equina* could not be ascertained in the present study because the experiments to measure the bactericidal properties of viable amoebocytes were confounded by the release of soluble factors from both the control and experimental cells upon treatment with Tween 20. As yet the nature and mechanism of action of the agent or agents responsible remain unknown. The large decrease in the SIs of the bacteria incubated with Tween 20-treated amoebocytes indicates that the response could involve a lysis. One of the best-known lytic agents in coelomate body fluids is lysozyme. This appears to be present in a wide range of phyla and is widely believed to aid in the nonspecific antibacterial defenses of both vertebrates and invertebrates (Millar and Ratcliffe, 1994). There have been comparatively few reports of lysozyme in acoelomates, although Phillips (1963) has described a series of experiments which indicate that lysozyme-like enzymes occurs in the mucus of *A. elegantissima*. Our assays with *A. equina* ALS did not, however, reveal lysozyme-like activity, as determined by lysis of *M. luteus* cell walls in *A. equina* amoebocytes. Thus the presence or absence of lytic agents in anthozoan amoebocytes remains equivocal. We are undertaking detailed characterization of the antibacterial activity in *A. equina* to establish the biochemical identity, cellular location, and biological effect of the factor or factors involved.

Acknowledgments

This work was supported by an NERC studentship award (G4/93/18/A) to DMCH.

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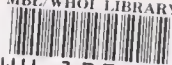
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